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## NUCLEIC ACID OF PROTEINS OF *VIBRIO CHOLERÆ* AND RELATED ORGANISMS.

BY

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[Received for publication, February 22, 1936.]

Two types of proteins have been identified in cholera vibrios by the simplified method of racemization of Woodman (1921). Under the influence of N/2 alkali one group of vibrio protein is found to have a smooth curve of racemization with an initial specific rotation (temperature 30°C.) of  $-80^{\circ}$  and a final rotation of  $-19^{\circ}$  after an interval of about 216 hours, whereas the other has a different curve of racemization beginning at  $-74^{\circ}$  and ending at  $-14^{\circ}$  at the same time interval. Those proteins with an initial rotation of  $-80^{\circ}$  and the final rotation of  $-19^{\circ}$  have been designated as protein I, and those corresponding to the other curve of racemization with comparatively smaller specific rotation values have been termed protein II (Linton, Mitra and Shrivastava, 1934). Neither an elementary analysis of the proteins nor the distribution of nitrogen by the method of van Slyke can differentiate the vibrio proteins, a fact which points to the conclusion that the two proteins are made up of the same constituents grouped together within the protein molecules in different ways. This has in fact been found to be the case. The comparative study of the two types of vibrio proteins by the method of racemization has now made it clear that certain amino acids are differently distributed in respect to their configuration in the two proteins (Mitra, 1936).

The study of nucleic acid of vibrio proteins was primarily undertaken with a view to their classification and was limited to the examination of the pyrimidine derivatives, since a complete analysis of all the constituents of the nucleic acid was difficult because of the amount of the acid available being very small. The yield of the nucleic acid from the proteins of the cholera vibrios was only 0.15 per cent.

Nucleic acid was prepared from both the types of proteins separately and the acids obtained in both the cases were subsequently mixed together for the analysis of their pyrimidine derivatives.

## Preparation of nucleic acid :—

Four hundred grammes of protein I were treated with four litres of 1 per cent NaOH, and kept in the incubator for two hours with occasional stirring. The fluid content was thereafter centrifuged in the Sharple's supercentrifuge and the clear brown solution after cooling below 20°C. was rendered very slightly acid to litmus with glacial acetic acid. It was then concentrated *in vacuo* at 60°C. to about one-third the original volume of the solution, and allowed to stand overnight in the ice-box. The solution was centrifuged again in the Sharple's supercentrifuge, and an equal volume of 95 per cent alcohol containing 2 c.c. of concentrated HCl was then slowly added to the clear fluid with constant stirring. During this operation the solution was maintained cold. After the precipitate of nucleic acid had settled down, it was recovered by centrifugalization, washed with alcohol twice and then with ether, and finally dried in a vacuum over sulphuric acid.

When the residual protein was similarly treated with alkali for a second time, it was found that the yield of nucleic acid was negligibly small. The total yield of the acid from 400 g. of protein I was 0.54 g. It was light brown in colour and the biuret test was negative. A very small quantity of the material was boiled in a test-tube with 10 c.c. of 10 per cent  $\text{H}_2\text{SO}_4$  over a flame, and the tests for purine bases, phosphate and carbohydrate were all found to be strongly positive. It was thus evident that the substance in hand was nucleic acid.

In the same way 0.66 g. of nucleic was obtained from 400 g. of protein II. The total quantity of the acid from both the types of proteins was 1.2 g., which is 0.15 per cent of the total protein.

## EXPERIMENTAL.

One gramme of nucleic acid was hydrolysed under reflux with 10 c.c. of 25 per cent sulphuric acid in an oil-bath at 125°C. for six hours.

The products of hydrolysis were diluted with water to 50 c.c. and treated while hot with an excess of a saturated solution of barium hydroxide to remove both phosphoric and sulphuric acids. The precipitate of barium salt was then filtered off and repeatedly washed with hot water. The excess of barium hydroxide was removed by saturating the solution with  $\text{CO}_2$ , and the solution was next decolorized with animal charcoal, filtered and concentrated *in vacuo*. The solution was then very slightly acidified with concentrated nitric acid, and treated in the cold with a sufficient quantity of silver nitrate solution to precipitate all the purine bases, which were filtered off. The silver nitrate solution was added until a test drop of the solution in contact with saturated barium hydroxide solution produced a yellow precipitate. The filtrate from the purine derivatives was then treated with cold saturated baryta solution till it was only faintly alkaline when the pyrimidine silver compounds precipitated out and were filtered off by suction and washed with water.

This silver compound precipitate was taken up in water, slightly acidified with sulphuric acid, heated to 40°C. and was treated with sulphuretted hydrogen to remove the silver. The sulphuric acid present in the filtrate containing the pyrimidine derivatives was exactly removed by means of baryta. The solution was finally concentrated *in vacuo* to a volume of 25 c.c.

*Cytosine* (6-amino-2-oxypyrimidine)  $C_4H_5N_3O$ .—The final solution was rendered acid with 0.2 c.c. concentrated  $H_2SO_4$ , and phosphotungstic acid solution was then slowly added, carefully avoiding the use of a large excess of the reagent. The phosphotungstic acid precipitate of cytosine was filtered off and washed with cold water. It was next suspended in water and the phosphotungstic acid removed by means of baryta and was filtered off. The excess of barium hydroxide in its turn was removed by means of  $CO_2$ . The solution of cytosine was evaporated nearly to dryness *in vacuo*, when it responded to the characteristic colour test of Wheeler and Johnson (1907). Cytosine was purified by crystallizing from water and finally washed with alcohol and ether and dried *in vacuo* over sulphuric acid. The yield was 0.022 g. with a melting point of  $320^\circ C.$  to  $322^\circ C.$

*Uracil* (2, 6-dioxypyrimidine)  $C_4H_4N_2O_2$ .—The filtrate from the phosphotungstic acid precipitate of cytosine was treated with baryta to remove from it phosphotungstic acid, and the excess of barium hydroxide was removed by saturating with  $CO_2$ . The solution was concentrated to about 5 c.c. and might contain both uracil and thymine, and possibly no cytosine, since cytosine is quantitatively precipitated with an excess of phosphotungstic acid (Kossel, 1896-97). The solution gave a strong colour test (Wheeler and Johnson, *loc. cit.*), thus indicating the presence in it of uracil. The absence of thymine was demonstrated in a way to be described below. Uracil was purified by crystallizing it from hot water, and finally washed with alcohol and ether and dried *in vacuo* over sulphuric acid. The yield was 0.018 g. with a melting point of  $334^\circ C.$  to  $336^\circ C.$

*Thymine* (5-methyluracil)  $C_5H_6N_2O_2$ .—This derivative was examined in the same solution of uracil by Johnson and Baudisch's (1921) colour reaction. A portion of the solution in presence of ferrous sulphate and acid sodium carbonate was heated under reflux for a few minutes and then the acetole ( $CH_3COCH_2OH$ ) that might have been formed from thymine, if any thymine was present, was distilled off. The test was carried out by attempting to prepare with o-amino-benzaldehyde in an alkaline solution of acetole its 3-oxyquinaldine compound which is characterized by its blue fluorescence, and was found negative.

A portion of cytosine, too, was tested for thymine in the same way, and the same negative result was obtained.

### CONCLUSION.

The presence of cytosine and uracil and the absence of thymine in the nucleic acid of the vibrio proteins point to the conclusion that the acid has the pyrimidine constitution of a plant and not of an animal nucleic acid. It should be noted that the nucleic acid examined was a mixture of approximately equal quantities of the acids prepared from the two types of vibrio proteins.

### SUMMARY.

1. Nucleic acid was prepared from proteins of cholera and cholera-like vibrios by treating the powdered proteins with 1 per cent caustic soda solution at  $37^\circ C.$  for two hours.
2. Cytosine and uracil were isolated from the products of acid hydrolysis of the nucleic acid, and thymine was found absent.

I wish to express my thanks to Dr. R. W. Linton, PH.D., Officer-in-Charge, Cholera Inquiry, All-India Institute of Hygiene and Public Health, Calcutta, for suggestions throughout the course of this investigation.

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## THE ABSORPTION SPECTRA OF THE PROTEINS OF *VIBRIO CHOLERÆ* AND RELATED ORGANISMS.

BY

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THE method of racemization as developed by Woodman (1921) was previously employed in order to differentiate the vibrio proteins. By this method only two types of proteins designated as protein I and protein II were identified amongst 60 to 70 strains so far examined (Linton, Mitra and Shrivastava, 1934 ; Linton, Shrivastava and Mitra, 1935). To substantiate this finding, the optical behaviour of the amino acids released after acid hydrolysis of the racemized proteins I and II was compared and it was found that a considerable difference in the spatial arrangement of the amino acids in the protein molecules of the two types does exist (Mitra, 1936). In the course of this investigation it was thought advisable to study the absorption spectra of the vibrio proteins for their differentiation.

### APPARATUS.

The apparatus employed was a quartz ultra-violet spectragraph of Adam Hilger, Ltd., and was used in conjunction with a rotating sector photometer. The spectragraph contained a wave-length scale mounted inside it. For the emission spectrum copper electrodes were used.

### METHOD.

Simultaneous adjacent pairs of photographs of the spark across the electrodes were taken, one through a cell containing the solution of which the absorption coefficient was desired and the other through a cell containing the solvent. A number of such pairs of photographs were taken at different graduations of the sector. The photographic plate was then developed and dried. Those points where the intensity of light passing through the solution and the solvent were of equal value were noted, and the absorption of the solution for the wave-length

corresponding to those points were measured. A whole absorption band could thus be obtained when the absorption coefficients were plotted against wave-lengths.

The absorption coefficient  $\epsilon$  was calculated from the following formula :—

$$\epsilon = \frac{1}{cd} \cdot \log \frac{I_o}{I_t}$$

where

$c$  = concentration of the solution expressed in percentage,

$d$  = thickness of the absorbing layer in centimetres,

$\log I_o$  = logarithm of the wave-length of the incident light,

and  $\log I_t$  = logarithm of the wave-length of the transmitted light.

The Adam Hilger sector photometer was graduated to read in terms of  $\log_{10} \frac{I_o}{I_t}$ .

#### PREPARATION OF PROTEINS.

Proteins derived from six vibrio strains, each representing one of the six chemical groups (Linton and Mitra, 1934), were studied. About 25 grammes of the bacterial mass were taken up in water, dissolved by the addition of the minimum amount of alkali, and centrifuged free from other impurities. To this clear fluid acetic acid was added to neutralize the excess of alkali, and the globulin separated out by repeated precipitations with half-saturated ammonium sulphate. The globulin was then dialysed against running water followed by distilled water until it was free from salts. The pseudoglobulin that remained in solution was separated from the euglobulin by filtration. The pseudo- and euglobulins were then precipitated with alcohol, washed and dried.

The vibrio strains selected for the examination of their proteins were 1617, E, 603, W 880, 676 and Basrah 4 respectively. As determined by racemization the first three contain protein I and the last three protein II. The Table below gives their origin, protein and specific carbohydrate type and also their chemical group :—

TABLE.

Strain.	Origin.	Protein number.	Carbohydrate number.	Chemical group.
1617 ..	Cholera	I	I	I
E ..	Cholera	I	II	II
603 ..	Carrier	I	III	VI
W 880 ..	Water	II	II	III
676 ..	Contact	II	I	IV
Basrah 4 ..	Cholera	II	III	V

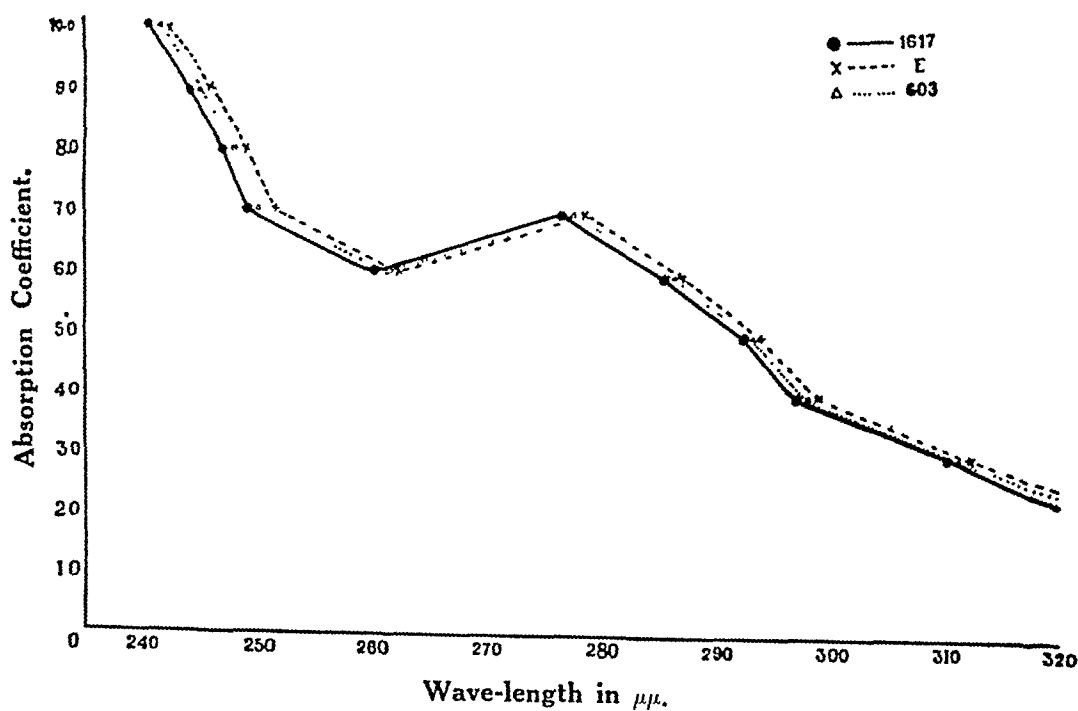
The pseudoglobulin fractions of the proteins derived from these strains were each taken up in N/20 NaOH ( $\text{Na}_2\text{CO}_3$ -free) and their absorption coefficients were measured. The concentrations of the protein solutions used were either 0.1 or 0.125 per cent. The protein solutions of 1617 (protein I) and W 880 (protein II) were given more intensive study. After the preliminary readings they were placed in an incubator at  $37^\circ\text{C}$ . in order to racemize the proteins, and measurements of their absorption spectra were taken at intervals.

### RESULTS.

The accompanying diagrams will illustrate the results obtained.

All the three proteins I before incubation at  $37^\circ\text{C}$ . possess identical absorption curves (Graph 1) and no variation could be observed among them. The same is

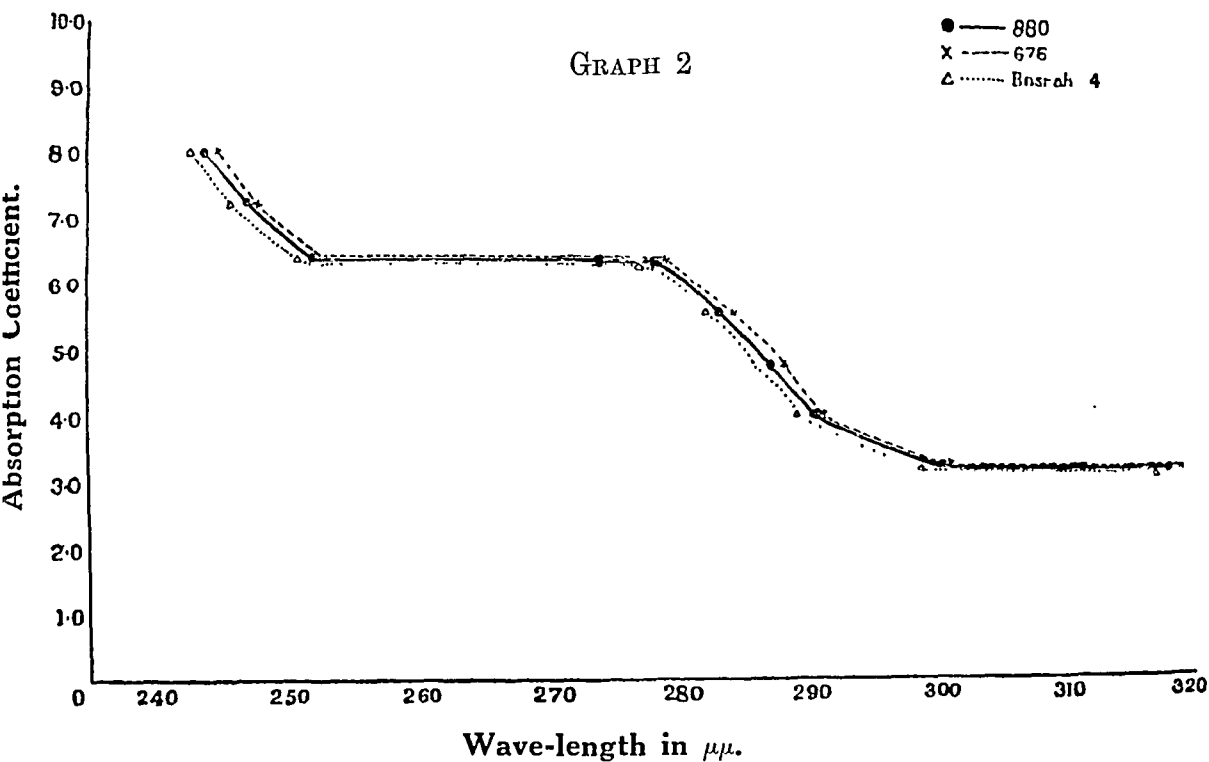
GRAPH 1.



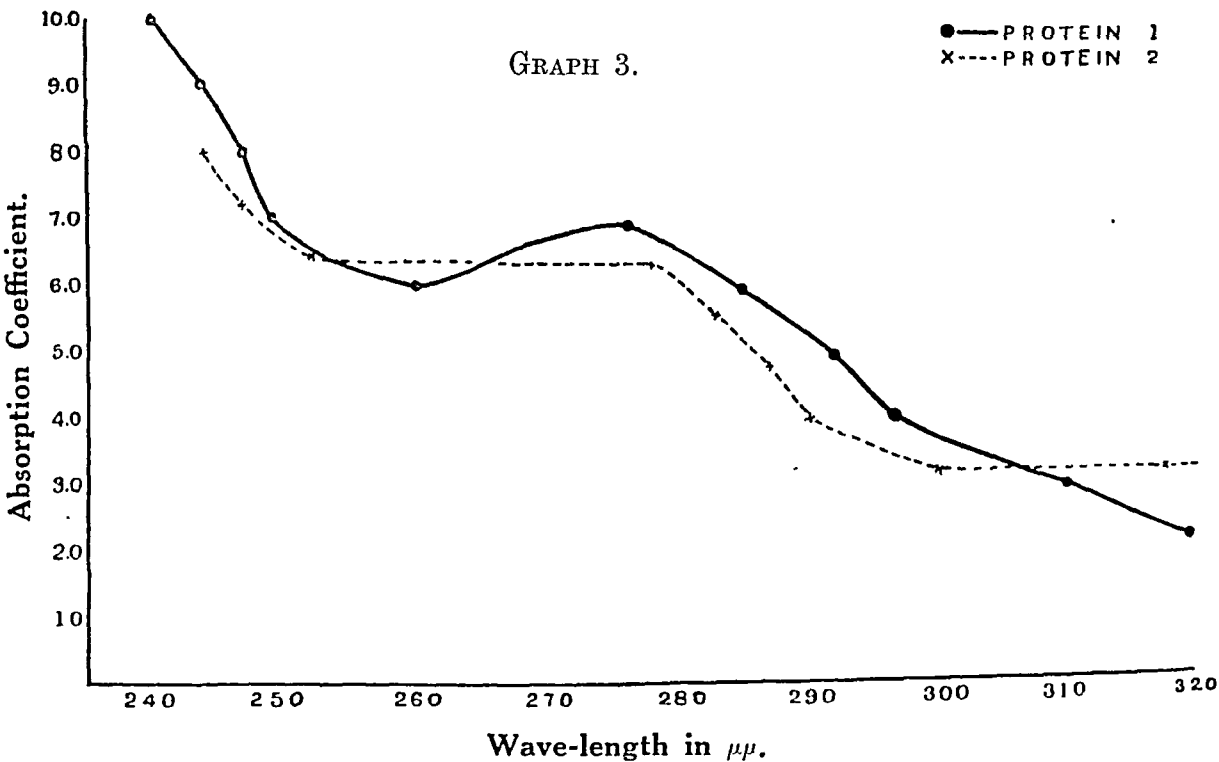
GRAPH 1.—Absorption curves of vibrio proteins I in N/20 alkali before incubation.

true of the three proteins II (Graph 2). Evidently, the three proteins of type I are similarly constituted, and the three proteins of type II are also identical. It will be observed that the spectrum shifted a little during the course of the experiment, but the nature of the curves was unchanged.





GRAPH 2.—Absorption curves of vibrio proteins II in N/20 alkali before incubation.

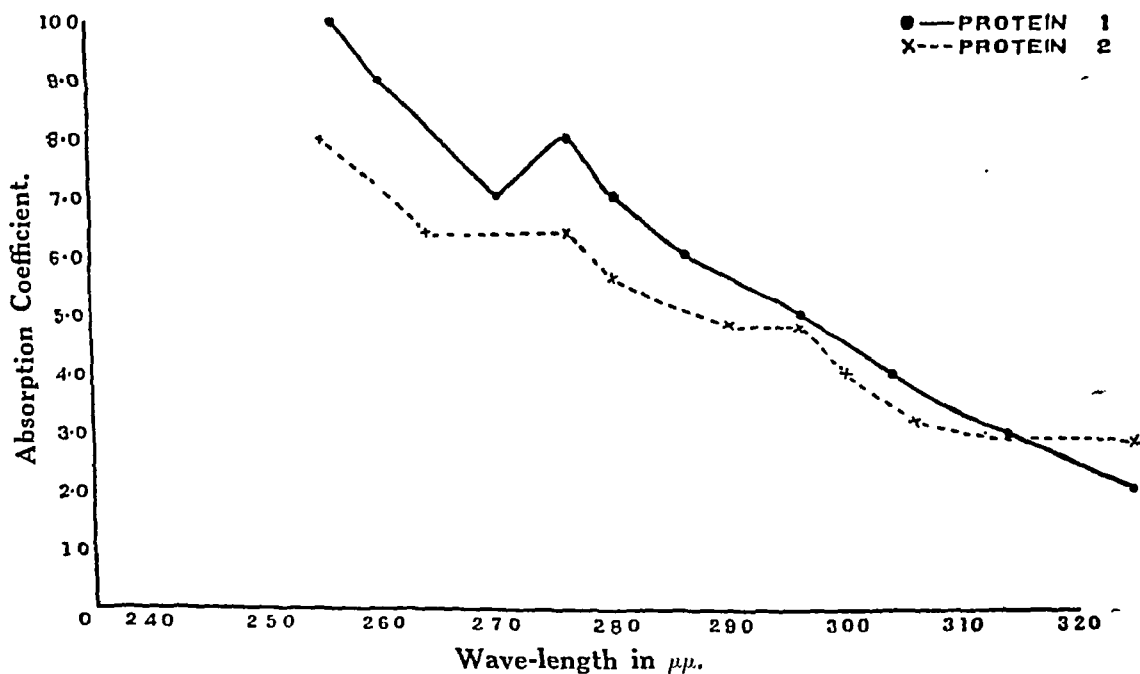


GRAPH 3.—Absorption curves of vibrio protein I (1617) and vibrio protein II (W 880) in N/20 alkali before incubation.

The two types of protein have distinctly different curves (Graph 3). Protein I seems to have a peak at about  $275\mu\mu$ , the absorption coefficient  $\epsilon$  being 7.0, whereas protein II possesses a flat curve between  $252\mu\mu$  and  $278\mu\mu$ ,  $\epsilon$  being 6.4. The two curves cross each other finally at about  $306\mu\mu$  where  $\epsilon = 3.2$ .

Racemization under the influence of such dilute alkali as N/20 is undoubtedly very slow and incomplete since even in N/2 NaOH the process takes about 250 hours to come to an end (Linton, Mitra and Shrivastava, *l. c. cit.*). Nevertheless, the absorption curves are clearly changed with the progress of racemization. On the fourth day of incubation (Graph 4), protein I has a peak at about  $275\mu\mu$  ( $\epsilon = 8.0$ ).

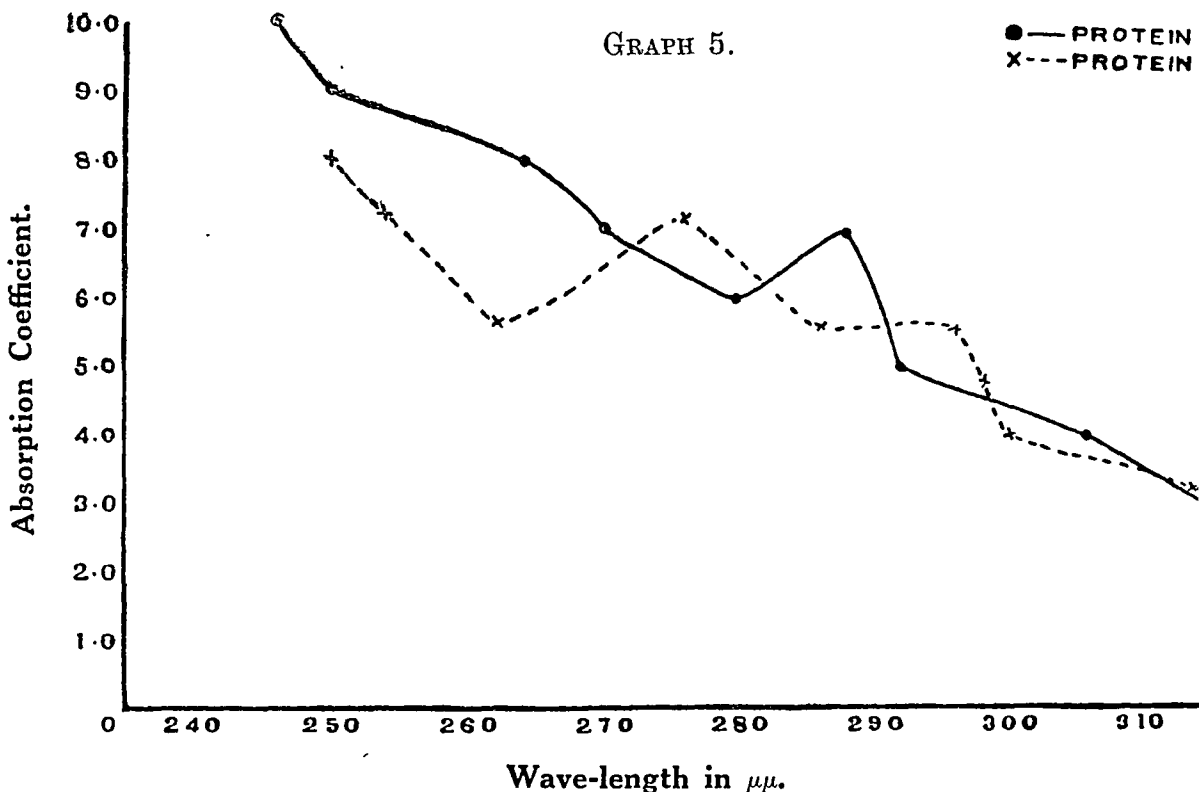
GRAPH 4.



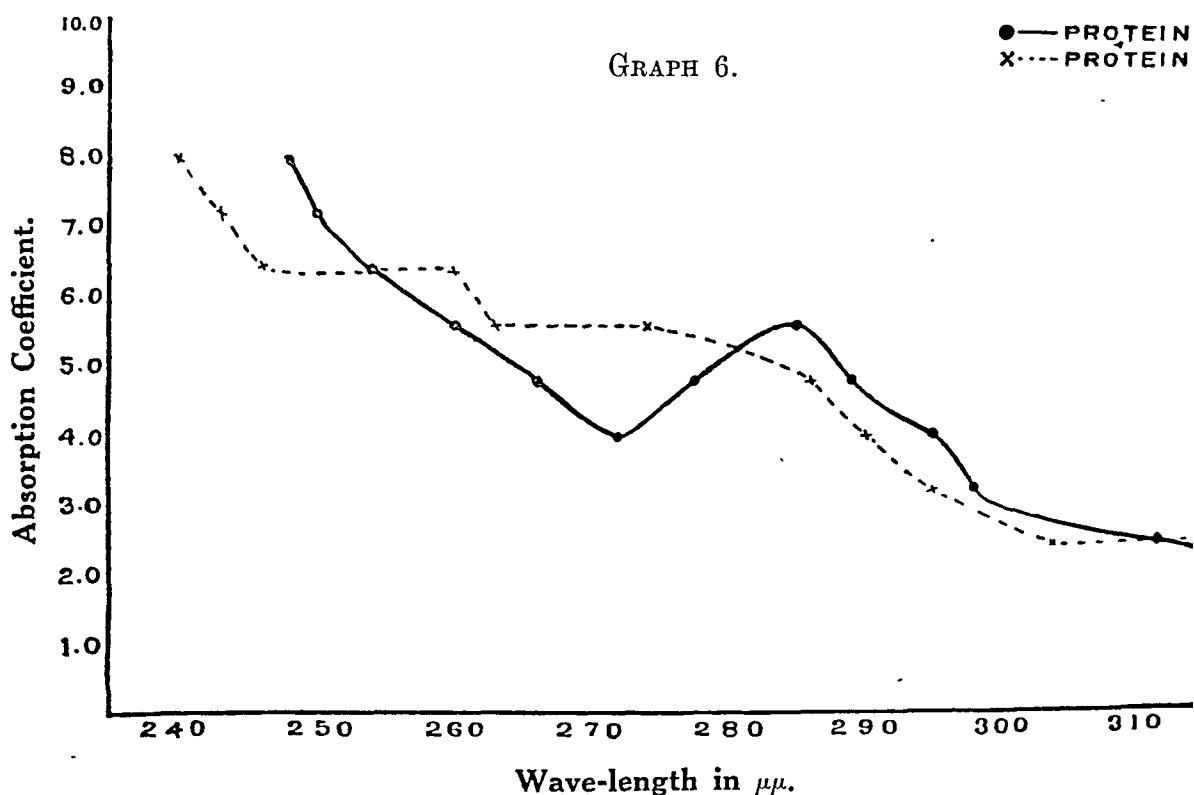
GRAPH 4.—Absorption curves of vibrio protein I (1617) and vibrio protein II (W 880) in N/20 alkali on the fourth day of incubation at  $37^{\circ}\text{C}$ .

whereas protein II exhibits two flat regions, one between  $264\mu\mu$  and  $276\mu\mu$  where  $\epsilon = 6.4$  and the other between  $290\mu\mu$  and  $296\mu\mu$  where  $\epsilon = 4.8$ . The two curves cross each other at about  $312\mu\mu$ ,  $\epsilon$  being 2.8.

On the seventh day of incubation (Graph 5), protein I exhibits a curve with a peak at about  $288\mu\mu$  ( $\epsilon = 7.0$ ), whereas protein II curve shows more peculiarity in having a peak at about  $276\mu\mu$  ( $\epsilon = 7.2$ ), and a straight region between  $286\mu\mu$  and  $296\mu\mu$  ( $\epsilon = 5.6$ ). Furthermore, the two curves cut each other in five places at wave-lengths about  $272\mu\mu$ ,  $282\mu\mu$ ,  $290\mu\mu$ ,  $298\mu\mu$  and  $312\mu\mu$  where the absorption coefficients are 6.8, 6.2, 5.6, 4.4 and 3.4 respectively.



GRAPH 5.—Absorption curves of vibrio protein I (1617) and vibrio protein II (W 880) in N/20 all on the seventh day of incubation at 37°C.

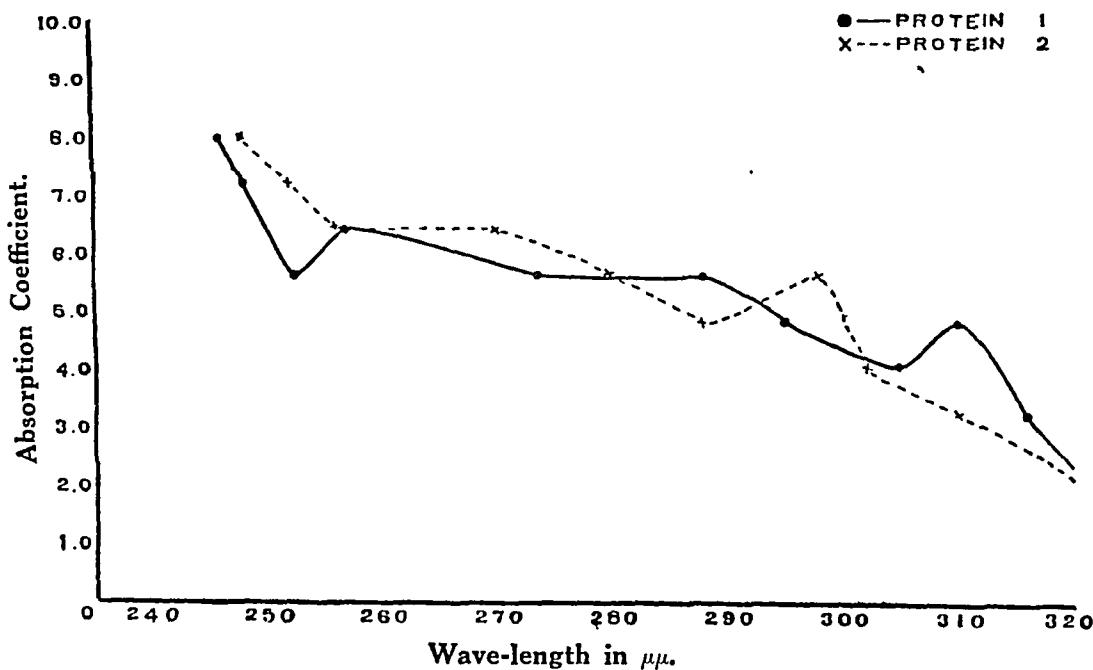


GRAPH 6.—Absorption curves of vibrio protein I (1617) and vibrio protein II (W 880) in N/20 all on the tenth day of incubation at 37°C.

On the tenth day of incubation (Graph 6), the peak in protein I curve seems to have shifted to  $286\mu\mu$  ( $\epsilon = 5.6$ ) and protein II has again two flat portions in its curve, one between  $246\mu\mu$  and  $260\mu\mu$  and the other between  $266\mu\mu$  and  $274\mu\mu$  corresponding to absorption coefficients  $6.4$  and  $5.6$  respectively. The two absorption curves cross each other at three points at wave-lengths  $254\mu\mu$  ( $\epsilon = 6.4$ ),  $280\mu\mu$  ( $\epsilon = 5.2$ ) and  $312\mu\mu$  ( $\epsilon = 2.4$ ) respectively.

On the fifteenth day of incubation (Graph 7), protein I has two peaks, one at about  $257\mu\mu$  ( $\epsilon = 6.4$ ) and the other at about  $310\mu\mu$  ( $\epsilon = 4.8$ ) respectively. Protein II has a flat portion between  $256\mu\mu$  and  $270\mu\mu$  ( $\epsilon = 6.4$ ) and also a peak at about  $298\mu\mu$  ( $\epsilon = 5.6$ ). The two curves cut at three points and touch at one near about  $258\mu\mu$  ( $\epsilon = 6.4$ ). The points of intersection are at about  $280\mu\mu$  ( $\epsilon = 5.6$ ),  $292\mu\mu$  ( $\epsilon = 5.2$ ) and  $302\mu\mu$  ( $\epsilon = 4.2$ ) respectively.

GRAPH 7.



GRAPH 7.—Absorption curves of vibrio protein I (1617) and vibrio protein II (W 880) in N/20 alkali on the fifteenth day of incubation at  $37^{\circ}\text{C}$ .

The absorption coefficients for waves longer than  $320\mu\mu$  are not plotted, because in these regions the absorption is continuous and no peculiarity is observed.

As the results show, the absorption curves of the two types of proteins change with time under the conditions of the experiment. This result is probably due to the change in the arrangement of the amino acids in the protein molecules which is being brought about by the process of racemization, and is a further indication that the two proteins are differently constituted.

After the fifteenth day of incubation the protein solutions of 1617 and W 880 began to show turbidity. Measurement of the absorption spectra was, therefore, not carried further.

The euglobulin fractions of the proteins were sometimes very difficult to dissolve in such alkali as dilute as N/20 and they were accordingly not examined. The observations were made with the pseudoglobulin fractions only. A stronger alkali, such as half-normal, could not be used as this medium would itself have absorbed all the light in the ultra-violet region and thus rendered the measurements of the absorption spectra impossible.

#### DISCUSSION.

The study of the absorption spectra of the vibrio proteins is apparently a suitable method for their differentiation and gives the same results as the method of racemization which is usually employed for the purpose. The failure to obtain more than two types of absorption curves of the vibrio proteins also shows that there are probably only two types of proteins in the vibrios studied. The differences observed either in the absorption or racemization curves of the vibrio proteins are quite significant, and it has previously been shown that the differences in the proteins of the vibrio group are in the differences in the spatial arrangement of the amino acids in the protein molecules of similar constituents (Mitra, *loc. cit.*).

#### SUMMARY.

The absorption curves of the vibrio proteins I are identical, and so also are those of vibrio proteins II.

The two types of proteins in the vibrios studied differ in their absorption spectra.

The differences in their absorption curves become more and more pronounced under the influence of N/20 alkali at 37°C.

#### ACKNOWLEDGMENTS.

I desire to express my grateful thanks to Dr. R. W. Linton, PH.D., Officer-in-Charge, Cholera Inquiry, All-India Institute of Hygiene and Public Health, Calcutta, for his encouraging interest and to Prof. J. C. Ghosh, D.Sc., of the University of Dacca, for his very kind co-operation in this work.

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## ON INHIBITION OF INDIVIDUAL TYPES OF CHOLERA BACTERIOPHAGE BY VIBRIO EXTRACTS.

BY

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THAT suitably prepared bacterial extracts inhibit the action of bacteriophages was shown by Levine and Frisch (1934) and Burnet (1934). Gough and Burnet (1934) later identified the phage-inactivating agent of bacterial extracts as a polysaccharide. It will be shown in this paper that similar inhibition with extracts of true cholera and allied vibrios is also found in case of some of the known types of cholera bacteriophages.

So far, 11 types of cholera bacteriophages have been isolated. These have been designated as types A, B, C, D, E, F, G, H, J, K and L. The general technique of isolation and identification of types was first described by Asheshov *et al.* (1933) working with types A, B and C. By further modifying the technique, other types were isolated by Dr. A. C. Vardon at this Institute (Morison, 1932). The types isolated in this Institute and designated as above, were used in this investigation, excepting type L, as difficulties were encountered in its propagation.

### TECHNIQUE.

In the study of inhibition we have adopted generally the technique described by Burnet (*loc. cit.*). The organisms were grown in Roux's flasks for 48 hours. The growth from each flask was washed and emulsified in 10 c.c. distilled water. The emulsions were placed in a water-bath at 55°C. for 72 hours. They were then diluted by adding twice the amount of distilled water, and filtered through a Seitz filter. This dilution was necessary as considerable difficulty was experienced in filtering crude extracts through Seitz filters. Burnet filtered his extracts through gradacol membranes as he found that Seitz filtrates proved inactive and Berkfeld filtrates very much weaker. We found that filtration through L<sub>3</sub> candles made the extracts almost inactive. As gradacol membranes were not available, Seitz filtrates were used, which, however, gave good results.

# 16 The Inhibition of Individual Types of Cholera Bacteriophage.

whereas the extracted strain W 1232 which was resistant to all types, inhibited types K, L, P and H only. Six strains were resistant to A, G, K and L types and lysed by the rest. In spite of A resistance, A type of 'phage was inhibited by some of them. Other variations were similarly noticed. Thus the extracts of strains having a common 'phage resistance behaved differently. The same remarks hold good in case of extracts of strains resistant to one or more types. It would appear that the so-called 'contrary findings' noted by Burnet, were quite common in this series, and that there was no correlation in general between 'phage resistance of strains, and the inhibition of types with their corresponding bacterial extracts. It, however, appears that 'phage resistance does influence inhibition to some extent for when a strain is resistant to a number of types, the inhibition noted with its extract is limited, e.g., strains 1612, Baara II, W 3075 and El Tor I.

## INHIBITION OF 'PHAGE TYPES AS A POSSIBLE METHOD OF ASCERTAINING THE NATURE OF POLYSACCHARIDES IN STRAINS OF VIBRIOS.

It was shown by Gough and Burnet (*loc. cit.*) that the 'phage inactivating agent present in the extracts was a polysaccharide. They also showed that this polysaccharide of living bacterial cells (obtained in such autolysates) was more complex than those obtained in the usual hapteno preparations. While working with pneumococcal polysaccharide, similar findings were recorded by Avery and Goebel (1933). On these findings, Gough and Burnet (*loc. cit.*) have supported the suggestion of Layme and Frisch (1934) that 'phage inhibiting activity' adds a useful tool to the armamentarium of those investigating the chemical structure of bacteria.

The variations in inhibition, which we have noted with the extracts of strains studied, could therefore be attributed to a corresponding difference in the nature of their polysaccharides. It was decided to see whether, by means of some common inhibition factor, the strains could be classified into distinct groups and then to compare such groups with the groups already determined by Linton *et al.* (1934, 1935) by other chemical analysis, i.e., by hapteno preparations.

It was found that the strains could be grouped together as follows:—

Inhibition of 1	no types always	ing A type e.g.
extracts of strains	1612, 1613, 1614	8, 11, 15, 8, 11
1612, 1613, 1614		

Inhibition by	is or less fast	e.g. Baara II
extracts of strains	1615, 1616	
1615, 1616		

Inhibition of 2	no types e.g.	1617, 1618
extracts of strains	1617, 1618	
1617, 1618		

Inhibition of 3	no types e.g.	1619, 1620
extracts of strains	1619, 1620	
1619, 1620		

Inhibition of 4	no types e.g.	1621, 1622
extracts of strains	1621, 1622	
1621, 1622		

Inhibition of 5	no types e.g.	1623, 1624
extracts of strains	1623, 1624	
1623, 1624		

Inhibition of 6	no types e.g.	1625, 1626
extracts of strains	1625, 1626	
1625, 1626		

agent

same range of inhibition as was observed with the extracts of group I strains. It might, therefore, be concluded that there is some progressive change in the nature of polysaccharides of the strains listed in these three groups.

As a control observation, extracts of three strains, one from each group, were then tested to determine whether in the process of routine extraction as described above, quantitative differences in their polysaccharide content were noted. This was necessary to show that variations in inhibition were not due either to the different concentrations, or total absence of polysaccharides in such extracts. The reducing substance contents in terms of glucose of these extracts were tested and also the contents on hydrolysis for 1 hour with 3 per cent sulphuric acid. The results are given in Table III:—

TABLE III.

	REDUCING SUBSTANCE CONTENT OF EXTRACTS.	
	Before hydrolysis.	After hydrolysis.
Group I, 1617 ..	0.06 per cent	0.25 per cent
Group II, W 880	0.06 per cent	0.23 per cent
Group III, 603 ..	0.07 per cent	0.3 per cent

It will be seen from this table that with the routine method of extraction not much difference was noted in the polysaccharide contents of the strains analysed.

TABLE IV.

Groups by inhibition.	Strains.	Classification by polysaccharide types (Linton).	Strains.
I .. {	1617 Inaba	I (polysaccharide) type I	1617 Inaba 1612 El Tor I
II .. {	Rangoon recovered Cholera 603 Basra II 505	III (polysaccharide) type III	Rangoon recovered Cholera 603 Basra II
III .. {	(a) W 3075 1612 (b) W 880 El Tor I	II (polysaccharide) type II	3075 1612 W 880 El Tor I



In Table IV a comparison is attempted between our classification of vibrios by inhibition and that determined by Linton according to their respective polysaccharides after chemical analysis. The strains of which the polysaccharide types were determined by Linton are only listed in this table.

It will be seen from this table that groups by inhibition correspond to a certain extent only with the groups determined by Linton. There are three exceptions: strain 1612 falls in inhibition with group III, though its polysaccharide is of Linton type I. The second exception was noted with the El Tor I strain. The extract of this strain did not inhibit any of the types while its polysaccharide was of type I chemically. While there is thus general agreement in both the groupings, some differences as noted above are quite likely to occur.

The strains 1612 and El Tor I were both found to be resistant to a number of types when compared with other strains. Such 'phage types resistance will affect the bacterial component brought out in extracts which is associated, as stated by Burnet, with 'phage inhibition.

It appears, therefore, that extracts prepared from a true cholera vibrio will inhibit at least some of the known types of cholera bacteriophages. It also appears that by inhibition, the polysaccharide nature of strains can also be determined to some extent. However, a number of strains will have to be examined before this point is finally determined. There is thus a possibility of utilizing 'inhibition' for the study of vibrio strains in the field in order to determine their polysaccharide nature.

#### SUMMARY.

It is shown that extracts of strains of true cholera vibrios inhibit some of the 10 types of cholera 'phages used in this investigation. No definite correlation was obtained between 'phage type resistance and corresponding type inhibition, though multiple type resistance was associated with a diminution in the number of types inhibited. The extracts investigated were classified into three groups according to their 'phage type inhibitions. These groups were found to be generally similar to the groups obtained by Linton according to the respective polysaccharides of those strains determined by chemical analysis.

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## AGGLUTINATION IN THE VIBRIOS.

### Part I.

#### THE EFFECT OF HEAT ON CHEMICAL STRUCTURE AND SURFACE POTENTIAL.

BY

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IN the study of which this is the first part, physical and chemical methods have been applied to the problems of vibrio agglutination. In this paper we are presenting our results with respect to the effect of heat, and later communications will deal with the rôles played by salts and by different types of normal and immune sera.

The study of the effect of heat is of particular interest because current serological practice in the vibrios is based on the double antigen hypothesis, in which the bacteria are considered to consist of two portions, one heat stable and the other heat labile. Various workers have found that the heat labile or 'H' antigen of the vibrios can be destroyed by placing the organisms for two hours at about 100°C., while the stable or 'O' portion is unchanged by this treatment, and Gardner and Venkatraman (1935) have put forward a classification of the vibrios based on the differential serological reactivity of the two portions.

No work so far reported has included any attempt to ascertain what changes are actually brought about in the vibrios by heat, and it was accordingly of interest to do so, and to attempt a physical and chemical explanation for the serological changes which occur. It follows from the double antigen hypothesis that the 'H' antigen, either whole or disintegrated, is to be found in the surrounding fluid after boiling, while the material remaining in the organisms themselves represents the 'O' antigen. By comparing the chemical analyses of the boiled organisms with the material in the supernatant fluid and with the whole unheated vibrios it should be possible to arrive at some understanding of the meaning in physico-chemical terms of the phrase 'destruction of the "H" antigen', and also of the constituents which remain to form the 'O' antigen.

## A. THE EFFECT OF HEAT ON CHEMICAL STRUCTURE.

The work in this section has been carried out on one strain, Rangoon Smooth, which is a typical vibrio of chemical group I, isolated from a non-fatal case of cholera in Rangoon in 1933. The characteristics of this strain have been given in detail elsewhere (Linton, Shrivastava and Mitra, 1935).

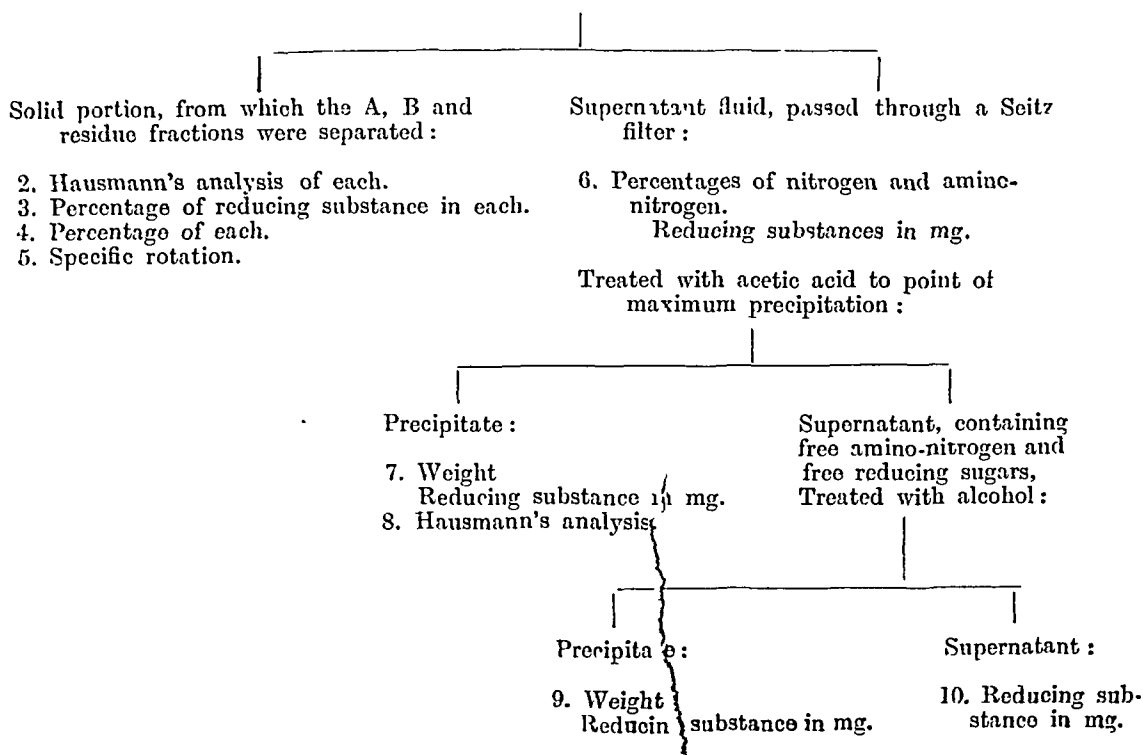
The method consisted essentially of heating the organisms for various periods and of studying separately the solid portion and the supernatant fluid. The solid portion was further separated into the A, B and residue fractions which we have already described (Linton and Mitra, 1934; Linton, Mitra and Seal, 1935) in order to determine the effect of heat on these constituents. The scheme of analysis is given in the following diagram, in which the numbered determinations correspond to the numbered sections of the 'Results'.

The forty-eight-hour growth on agar was washed off in 0.5 per cent phenol and the organisms freed from the agar contamination by repeated washing with buffer at pH 7.0 through a Sharple's supercentrifuge. Heavy suspensions of the organisms were then made in the buffer or in normal saline and heated in boiling water or at 37°C. for two hours or at 56°C. for four hours.

### *Scheme of analysis followed in studying the effect of heat on the vibrios.*

Forty-eight-hour growth on agar washed free from reducing substances and used unheated or heated for varying periods in buffer solution of pH 7.0 or in normal saline.

#### 1. Weight of reducing substances in the whole material.



The periods in the boiling water-bath were 15 minutes, one-half hour, one hour and four hours. The suspensions at all periods save the one-half hour period were in the buffer solution. At one-half hour the organisms were in normal saline. It is evident from the results in the following tables that whether buffer or NaCl was present made no appreciable difference in the progressive changes which occurred. The time intervals were chosen to cover the period during which the 'H' antigen was being destroyed. The period of four hours at 56°C. was taken to follow the changes occurring in vibrios during the agglutination reaction; normal saline was used here as in the usual agglutination test; the two-hour period at 37°C. was taken for the same reason, as this period has been used by some workers.

### Results.

(1) Aliquot portions of untreated and variously heated organisms together with their supernatant fluids were tested for amount of reducing substance by boiling them for two hours in 3 per cent  $\text{H}_2\text{SO}_4$ . The results are given in Table I:—

TABLE I.

*The effect of heat on the amount of reducing substance in vibrios and their supernatant fluids.*

Treatment.			Weight of bacteria, g.	Weight of reducing substances, g.	Per cent.
Unheated	..		17.5	0.390	2.2
15 min. 100°C.	..		17.6	0.340	1.9
$\frac{1}{2}$ hr.	..	..	15.7	0.322	2.0
1 "	..	..	15.1	0.300	2.0
4 hrs.	..	..	14.9	0.310	2.0
4 "	..	56°C.	15.0	0.315	2.1
2 "	..	37°C.	16.1	0.315	1.9

It is evident from Table I that the total amounts of polysaccharide are unchanged. Later work (Table VIII) will, however, show that, while destruction of polysaccharide does not occur to any appreciable extent, there is a marked difference in its distribution between the vibrios and the supernatant fluid as heating continues.

## THE SOLID PORTION (2, 3, 4 AND 5).

The solid portion was separated from the supernatant in the centrifuge, and the A, B and residue fractions prepared as described in the papers already referred to. These fractions have already been analysed in respect to their protein constitution, specific rotatory power, content of polysaccharide, and serological reactivity. The effect of heat is given in Tables II and III:—

TABLE II.

*Effect of heat on the A, B and residue fractions of vibrios as shown by Hausmann's analysis.*

Fraction.	Treatment.	N, per cent.	Amide N, per cent.	Humin N, per cent.	Total bases, per cent.	Total phos. filtrate, N, per cent.	Total N, per cent.
A	Unheated ..	14.0	15.0	1.4	18.8	65.4	100.6
	15 min. 100°C.	13.6	15.6	1.4	20.5	63.8	101.3
	1 hr. „ ..	13.3	14.2	1.0	18.0	68.4	101.7
	4 hrs. „ ..	13.7	15.0	1.3	19.9	62.6	98.7
B	Unheated ..	13.3	6.3	3.8	23.1	64.9	98.1
	15 min. 100°C.	13.0	6.9	3.3	24.1	67.8	102.1
	1 hr. „ ..	13.3	6.2	3.1	23.0	67.9	100.2
	4 hrs. „ ..	13.3	6.5	3.2	23.3	67.0	100.0
Residue	Unheated ..	13.8	7.2	3.5	22.1	68.3	101.2
	15 min. 100°C.	13.0	5.7	3.8	23.6	67.8	101.0
	1 hr. „ ..	12.6	5.7	4.0	24.0	65.8	99.6
	4 hrs. „ ..	11.9	4.9	7.5	26.2	61.2	99.8
Whole un- heated protein.	..	14.6	6.6	4.5	25.5	64.0	100.6

The unheated A fractions show the same characteristics as we have previously noted, namely, an amide nitrogen content about twice as high as that found in the whole protein, and a humin nitrogen value of about half. In these and other respects which have been studied the B and residue fractions appear identical with the whole protein (Linton, Mitra and Shrivastava, 1934). It is evident from Table II that neither the A or B fractions are appreciably altered by heat even after four hours. The residue, on the other hand, shows progressive changes. The total percentage of nitrogen declines, the amide nitrogen becomes progressively

less, and at the same time the percentage of humin nitrogen is doubled. These variations indicate that changes are being brought about in the protein by heat, and that nitrogen is being given off and the linkages between the amino acids are being opened up. The process resembles a mild hydrolysis. That the A fraction is apparently unaltered during heating is of interest in view of the probable importance of this portion in serological activity, and at the same time the changes brought about by heat in the residue, which makes up over 80 per cent of the whole protein, are sufficient to bring about the altered serological specificity which is characteristic of the 'O' antigen.

Table III gives the data on further work with these fractions :—

TABLE III.

*Effect of heat on the yields, carbohydrate contents and specific rotations of A, B and residue fractions of vibrios.*

Fraction.	Treatment.	Yield, g.	Yield, per cent.	REDUCING SUBSTANCES.		Specific rotation*.
				Mg.	Per cent.	
A	Unheated ..	1.08	4.8	20.2	1.8	—13.7°
	15 min. 100°C.	0.82	4.0	19.4	2.3	—10.5°
	1 hr. „ ..	0.88	4.4	17.6	2.0	—12.0°
	4 hrs. „ ..	0.68	3.9	12.3	1.8	— 9.4°
B	Unheated ..	2.96	13.2	39.1	1.3	—47.5°
	15 min. 100°C.	1.52	8.0	21.8	1.4	—45.0°
	1 hr. „ ..	2.00	10.0	17.4	0.8	—49.5°
	4 hrs. „ ..	1.76	9.1	15.0	0.8	—40.0°
Residue	Unheated ..	18.50	82.0	8.6	0.04	—50.0°
	15 min. 100°C.	17.50	88.0	8.2	0.05	—52.5°
	1 hr. „ ..	17.10	85.6	12.0	0.07	—51.0°
	4 hrs. „ ..	16.90	87.0	5.0	0.03	—58.0°

\* In N/2 alkali (NaOH) after 2 hours at 37°C.

As heating continues, the yield of A fraction becomes less, and as Table II shows, this result is not due to destruction of A in the vibrios, and accordingly must result from its solubility in the hot water. As we have already noted, A is highly hygroscopic and hence soluble. The percentage of reducing substance in A remains constant although its amount lessens equally with the yield, indicating that the polysaccharide is likewise going into solution.

'A' fraction acts like a substance of fairly ready solubility and of high resistance to heat. In the heated organisms it is present in lessened amount but with the same constitution as in the unheated organisms. The specific rotation is gradually lowered during heating, and at the four-hour period falls slightly outside the range ( $-10.3^{\circ}$  to  $-14.8^{\circ}$ ) previously found. If this change is actual it would point to a structural rearrangement of A during heating.

'B' fraction undergoes the same general type of change as 'A'.

The yield of residue also decreases with heating, some of it going into solution and some of it, as Table II indicates, being destroyed. The percentage increase in the residue is only an apparent one, and results from the fall in the percentages of the other two fractions.

#### THE SUPERNATANT FLUIDS (6).

The supernatant fluid of the boiled organisms and of the unheated vibrios which had stood for an equivalent length of time was passed through a Seitz filter. These filtrates gave strongly positive biuret, Molisch, xanthoproteic and Millon's tests, and were negative for Voisenet's reaction for tryptophane, and in the nitroso-indol reaction. Tests for glycine, leucine, arginine, histidine and tyrosine in the free form were also negative. The amounts of total nitrogen, amino-nitrogen and reducing substance were ascertained, and the results are given in Table IV:—

TABLE IV.

*Total nitrogen, amino-nitrogen and reducing substances in filtrates of vibrios after treatment by heat.*

Treatment.	Total nitrogen, mg.	Amino-nitrogen, mg.	Reducing substances, mg.
Unheated ..	27	1	22
15 min. 100°C. ..	32	29	45
$\frac{1}{2}$ hr. ,, ..	32	28	60
1 ,, ,, ..	35	30	68
4 hrs. ,, ..	38	36	98
4 ,, 56°C. ..	35	32	79
2 ,, 37°C. ..	25	24	48

It is interesting to note in Table IV that a certain amount of nitrogen and of reducing substance is removed from the organisms simply by allowing them to stand overnight at 10°C. in buffer solution of pH 7.0. The almost total absence of amino-nitrogen at this time shows that solution, but not disintegration, is at work.

Heating does not cause more than a small further rise in the total nitrogen, but the amino-nitrogen is greatly increased especially in the first 15 minutes, when it rises almost to its maximum. Thereafter the increase is slight. It appears possible, from this observation, that whatever disintegrative changes occur are concerned largely with the surfaces of the vibrios.

At the same time, the polysaccharide undergoes a constant rate of removal from the vibrios, indicating that this constituent is being derived from all portions of the organism. This finding coincides with our previous experience in extracting polysaccharides from the vibrios.

Heating in saline at 56°C. and 37°C. was also shown to extract reducing substance and amino-nitrogen from the vibrios.

#### PROTEIN PRECIPITATE (7) AND HAUSMANN ANALYSIS (8).

The supernatant fluids in the various experiments were treated with acetic acid to the point of maximum precipitation, the precipitates collected, dried and weighed, and the data obtained which are given in Tables V and VI :—

TABLE V.

*Acetic acid precipitates of supernatant fluids of vibrios after heating.*

Treatment.	Weight of original suspension, g.	WEIGHT OF PRECIPITATE.		Reducing substances, mg.
		G.	Per cent.	
Unheated ..	17.5	1.14	6.5	3.0
15 min. 100°C. ..	17.6	1.22	6.9	20.0
$\frac{1}{2}$ hr. ..	15.7	1.05	6.6	20.0
1 " ..	15.1	1.63	10.0	26.0
4 hrs. ..	14.9	2.02	13.0	32.0
4 " 56°C. ..	15.0	1.65	10.0	36.0
2 " 37°C. ..	16.1	1.00	6.2	20.0

The data in Table V show that as heating continues increasing amounts of acid precipitable substance are removed from the vibrios, until at the four-hour period 13 per cent has been lost. Taken in connection with Table IV the figures in



the last column indicate that only about one-third to one-half the polysaccharide present in the supernatant is precipitated with acetic acid, the remainder existing either in a free form or dissolved out of the protein by the acetic acid, in which the vibrio polysaccharides are very soluble.

Table VI gives the data on the Hausmann analysis of the acetic acid precipitates :—

TABLE VI.

*Hausmann analysis of the acetic acid precipitates of vibrio supernatants after heating.*

Treatment.	Nitrogen, per cent.	Amide N, per cent.	Humin N, per cent.	Total phos- photungstic precipitate, N, per cent.	Total filtrate, N, per cent.	Total N, per cent.
Unheated ..	10.2	7.5	Trace	25.1	68.5	101.1
15 min. 100°C. ..	10.0	7.0	2.1	23.1	68.4	100.6
$\frac{1}{2}$ hr. „ ..	10.5	7.5	3.3	20.0	69.8	100.6
1 „ „ ..	10.9	8.2	3.8	18.2	70.0	100.2
4 hrs. „ ..	11.4	12.4	6.0	12.0	69.8	100.2
4 „ 56°C. ..	10.0	9.8	4.9	16.2	69.0	99.9
2 „ 37°C. ..	9.9	7.0	Trace	23.9	68.1	99.0

The material in this precipitate has about 10 per cent of nitrogen, and the protein is accordingly not identical with that of the whole vibrio, in which about 14 per cent is present. There is accordingly a differential solubility under the influence of heat, and some portions of the vibrio are more affected than others. The amide nitrogen is increased, and reference to Table II shows that this constituent is decreased in the whole vibrio, and it is probable that the latter is the source of the increase. The rise of humin nitrogen from a trace to 6 per cent is also indicative of protein disintegration, and this rise is marked not only here but in the residue also (Table II) indicating that breakdown is occurring in the vibrio itself as well as in the material which has been extracted by the heat. The figures for the total bases fall from 25 per cent in the unheated to 12 per cent in the four-hour sample, an observation which indicates, when taken in connection with the data in Table II, that only the bases in the supernatant fluids are being destroyed by heat, while those remaining in the organisms are unchanged.

As in the previous tables, the data in Table VI again indicate that the lower temperatures have correspondingly smaller effects on the protein.

#### ALCOHOL PRECIPITATE (9 AND 10).

After the removal of the acetic acid precipitate, the supernatant fluids still contained free amino-nitrogen and material which reduced Benedict's solution without previous hydrolysis. In order to study this portion, the supernatant was treated with alcohol and the observations made which are given in Table VII :—

TABLE VII.

*Alcohol precipitates of vibrio supernatants after heating.*

Treatment.	Weight, mg.	Reducing substance, mg.	Reducing substances in supernatants after alcohol treatment, mg.
Unheated ..	56	5	5
15 min. 100°C. ..	820	15	8
$\frac{1}{2}$ hr. " ..	No ppt.	..	32
1 " " ..	640	10	40
4 hrs. " ..	900	16	64
4 " 56°C. ..	No ppt.	..	48
2 " 37°C. ..	" "	..	13

As Table VII indicates, the amount of precipitate after boiling in buffer solutions increases with the time, as does the reducing substance. It is probable that some of this weight represents salts from the buffer solutions in which the vibrios had been heated. No precipitates were obtained with alcohol from supernatants in which sodium chloride was the only salt present. The figures again indicate that heating extracts polysaccharide from the vibrios, and the figures in the last column show that much of this polysaccharide is non-precipitable by alcohol.

From Tables I, IV, V and VII it is possible to determine the total amounts of polysaccharide lost during heating, and these data are given in Table VIII :—

TABLE VIII.

*Total amounts of reducing substance in supernatants after heating for various periods.*

Treatment.	Mg. in original suspension.	Total mg. in various filtrates.	Percentage lost by original suspension.
Unheated ..	390	38	9
15 min. 100°C. ..	340	88	25
$\frac{1}{2}$ hr. „ ..	322	112	34
1 hr. „ ..	300	142	47
4 hrs. „ ..	310	210	67
4 „ 56°C. ..	315	162	51
2 „ 37°C. ..	315	81	25

The data in Table VIII make it clear that large percentages of polysaccharide are lost by the vibrios during heating, and these amounts increase steadily as the heating is continued, until at the four-hour period over two-thirds of the polysaccharide has been transferred from the organism to the surrounding fluid.

In the vibrios heated for four hours at 56°C. and for two hours at 37°C. only the supernatants have been studied, and these data have been given in the preceding tables. During the periods of heating at these temperatures the vibrios are not inert or unaffected by the heat, but are undergoing changes the same in kind although less in degree than those occurring at 100°C. Heating at 56°C. for four hours is equivalent to one hour in the boiling water-bath, and two hours at 37°C. has the same effect on the organisms as 15 minutes' boiling. The losses of polysaccharide are 51 per cent and 25 per cent, and of total substance 10 per cent and 6 per cent, respectively. The heating also causes total amino and humin nitrogen to increase and the bases to decrease.

From these results it is evident that the vibrios in the agglutination reaction cannot be considered as inert particles floating in the anti-serum. On the contrary they undergo progressive changes even at 37°C. and the agglutination tube at the end of the reaction contains not alone the vibrios and the anti-serum but free and combined polysaccharides, and proteins in various stages of disintegration.

## B. THE EFFECT OF HEAT ON SURFACE POTENTIAL.

The relationship between surface charge and the agglutination reaction will be considered more fully in the succeeding papers of this series. We are concerned here

only with the effect of heat on this physical property. Since the work of Northrop and de Kruif (1921-22) surface charge ('potential difference', or *P. D.*) has been recognized as a factor of outstanding importance in bacterial agglutination, and it was accordingly of interest to determine what changes occurred in it during the destruction of the 'H' antigen and the appearance of 'O' antigen as the sole serological factor in the vibrios.

### *Method.*

After 18 hours' growth on agar the vibrios were washed off into conductivity water by gentle rotation of the culture tube. In the present experiments the organisms were not washed further, since we wished to study the surface charges under the usual conditions of the agglutination test, in which the organisms are not washed free from growth and disintegration products. Comparative tests were, however, made with organisms washed twice, and the differences found, while constant, were small. This point will be considered further in later work. The organisms were used at a concentration of 100 million per c.c., and the sodium chloride (Kahlbaum reagent quality) was added to make the concentrations noted in the experiments. The final pH of the solution was approximately 7.0.

The direct microscopic method was used to observe the velocity of migration under the influence of an impressed voltage. The cell was similar to that described by Northrop (1921-22), and was provided with two funnels fitted with stop-cocks, one at each side of the cell. A direct current potential was applied through non-polarizable copper-copper-sulphate electrodes, from a 100-volt accumulator. Observations were made with a magnification of 560 diameters. The current passing through the solution was recorded by a milliammeter, and the resistance of the solution was determined separately by means of a Wheatstone bridge. Artificial light was used to illuminate the microscopic field, and a thick-walled flask filled with water was interposed to prevent heating of the cell.

When a difference of potential is established between the ends of a cataphoresis cell, the particles move in the fluid, and this motion is referred to as cataphoresis. However, the surface layers of water that are in contact with the upper and lower glass surfaces will also show a drift in the electric field, because water takes on a charge, usually negative, when in contact with glass. Various investigators have found that the movement of the water itself is at a minimum at distances of one-fifth and four-fifths the depth of the cell from its upper surface. Water at these two levels is practically stationary, and the uncomplicated movement of the particles can be observed. The cell used had a depth of 0.88 mm., and the levels at which the microscope was focused were accordingly 0.176 mm. and 0.704 mm. from the upper surface.

In order to eliminate the effect of polarization and the motility of the vibrios, all observations were repeated with the current in the cell reversed. In every case the observations were also made at both the indicated depths of the cell, and the average of all these observations was taken to represent the true 'slip' of the organism under study. In all cases the readings agreed very closely. The temperature at which the observations were taken varied from 25°C. to 27°C.

The formula used in calculating the potential difference from the observed cataphoretic velocities is as follows:—

$P. D.$  in millivolts =  $13 \frac{\text{millimicrons per sec.}}{\text{volts per cm.}}$  approx.; the relationship between this formula and the Lamb-Helmholtz formula is fully discussed by Winslow, Falk and Caulfield (1923-24).

The salt concentrations were as indicated in the table and figures. In the later part of the work, cataphoresis at salt concentrations of 0.0187 N and 0.0375 N was omitted, as experience with a large number of strains showed in every case a linear relationship between the migration rate and the salt concentration, and it was accordingly sufficient for our purpose to obtain the data at the lower concentration of 0.0093 N and the higher concentrations of 0.075 N and 0.15 N. It should be noted that 'normal saline' corresponds, when carefully made up of pure sodium chloride, to a concentration of 0.15 N.

### *Results.*

The effect of washing on the surface potentials of living cultures was first studied, and the results are given in Table IX. Washing was carried out by centrifugalizing the organisms in conductivity water, resuspending them and repeating the centrifugalization. They were again suspended and their potentials determined at various salt concentrations. The figures in this and the succeeding tables indicate millivolts.

TABLE IX.

*The effect of washing on the potential difference in living vibrio cultures.*

Strain.	Treatment.	CONCENTRATIONS OF NaCl.				
		0.0093 N.	0.0187 N.	0.0375 N.	0.075 N.	0.15 N.
Rangoon Smooth.	Unwashed	-29.9	-24.2	-18.6	-12.2	-6.5
	Washed	-30.2	-24.6	-19.2	-13.0	-6.6
2027 ..	Unwashed	-20.8	-18.2	-11.7	-6.5	-2.6
	Washed	-21.8	-15.7	-12.0	-6.6	-2.0
505 ..	Unwashed	-26.0	-19.9	-15.0	-7.8	-1.6
	Washed	-27.4	-21.3	-15.3	-8.4	0.0
W 880 ..	Unwashed	-24.2	-21.0	-14.8	-11.0	-3.4
	Washed	-26.0	-23.0	-18.2	-12.0	-3.4
1617 ..	Unwashed	-22.1	-16.0	-11.7	-6.5	-2.8
	Washed	-26.8	-18.8	-12.7	-7.8	-3.4

It will be seen from Table IX that washing has almost no effect on the potential. In general there is a slight rise, but this is not constant and the differences may well be within the limits of error in the method. In this respect the vibrios are a marked contrast to the bacillus of rabbit septicæmia which was studied by Northrop and de Kruif (*loc. cit.*). In the unwashed condition at pH 4.4 this organism had a *P. D.* of  $-9.0$ , and this was increased to  $-18.0$  after one washing and to  $-27.0$  after two washings: further washings did not appreciably alter the potential.

Heating has been carried out both at  $60^{\circ}\text{C.}$  for one hour, and for periods from 15 minutes to four hours in the boiling water-bath. The results are presented in Tables X and XI:—

TABLE X.

*The effect of heating for one hour at  $60^{\circ}\text{C.}$  on the potential difference in the vibrios.*

Strain.	Treatment.	CONCENTRATIONS OF NaCl.				
		0.0093 N.	0.0187 N.	0.0375 N.	0.075 N.	0.15 N.
Rangoon Smooth.	Unheated	$-29.9$	$-24.2$	$-18.6$	$-12.2$	$-6.5$
	1 hr. $60^{\circ}\text{C.}$	$-34.2$	$-27.9$	$-23.0$	$-18.5$	$-14.8$
2027 ..	Unheated	$-20.8$	$-18.2$	$-11.7$	$-6.5$	$-2.6$
	1 hr. $60^{\circ}\text{C.}$	$-30.4$	$-24.7$	$-20.8$	$-16.9$	$-13.0$
505 ..	Unheated	$-26.0$	$-19.9$	$-15.0$	$-7.8$	$-1.6$
	1 hr. $60^{\circ}\text{C.}$	$-26.0$	$-21.6$	$-18.2$	$-14.9$	$-11.7$
W 880 ..	Unheated	$-24.2$	$-21.0$	$-14.8$	$-11.0$	$-3.4$
	1 hr. $60^{\circ}\text{C.}$	$-31.9$	$-26.2$	$-21.3$	$-16.4$	$-13.0$
1617 ..	Unheated	$-22.1$	$-16.0$	$-11.7$	$-6.5$	$-2.8$
	1 hr. $60^{\circ}\text{C.}$	$-28.6$	$-23.4$	$-19.5$	$-15.2$	$-11.7$
Rangoon Rough (1)	Unheated	$-36.1$	$-29.1$	$-23.1$	$-16.6$	$-8.5$
	1 hr. $60^{\circ}\text{C.}$	$-36.9$	$-33.0$	$-28.8$	$-26.0$	$-23.9$
Rangoon Rough (2).	Unheated	$-39.0$	$-31.4$	$-24.1$	$-19.7$	*
	1 hr. $60^{\circ}\text{C.}$	$-46.1$	$-40.3$	$-35.9$	$-32.1$	$-30.4$
El Tor ..	Unheated	$-26.2$	$-20.8$	$-13.9$	$-9.1$	$-3.9$
	1 hr. $60^{\circ}\text{C.}$	$-27.0$	$-23.9$	$-21.0$	$-19.5$	$-18.2$

\* No observable effect of current; high speed movement in all directions.

The results given in Table X show that heating at 60°C. for one hour causes a greatly increased surface potential, especially at the higher salt concentrations. Northrop and de Kruif (*loc. cit.*) showed that the critical potential for agglutination to occur in the presence of immune serum was in the zone from  $-13.0$  to  $+13.0$  millivolts, and later work has tended to narrow rather than broaden this zone. Taking the value of  $-13.0$  millivolts and studying the results obtained in 0.075 N salt, it is evident that heating to 60°C. for one hour causes the vibrios to assume a potential above the critical point, and accordingly makes them less capable of being agglutinated. In the cases of the two Rangoon Rough strains, the results show that even in the living condition these organisms are above the critical value, and the effect of heat is again to cause a large increase in potential difference.

These observations explain at least in part the results of Linton and Seal (1935) who showed that heating vibrios at 60°C. even for half an hour often either reduced or completely abolished their agglutinability. This result is understandable from the data given in Table X.

For the experimental work given in Table XI, the vibrios were taken up in distilled water and placed for the periods indicated in the boiling water-bath, and subsequently diluted with the saline solutions. The vibrios in these experiments were unwashed, since we wished to reproduce the conditions of the usual agglutination reaction, in which washing is not often used.

TABLE XI.

*The effect of heating at 100°C. for various periods on the potential difference in the vibrios.*

Strain.	Treatment.	CONCENTRATION OF NaCl.		
		0.0093 N.	0.075 N.	0.15 N.
Rangoon Smooth.	Unheated ..	-29.9	-12.2	-6.5
	15 min. 100°C.	-36.9	-20.5	-15.8
	1 hr. „ ..	-36.0	-19.6	-14.5
	2 hrs. „ ..	-37.0	-19.5	-15.6
	4 „ „ ..	-35.1	-20.0	-15.0
2027 ..	Unheated ..	-20.8	-6.5	-2.6
	15 min. 100°C.	-33.4	-16.1	-13.2
	1 hr. „ ..	-31.9	-17.4	-13.0
	2 hrs. „ ..	-32.5	-16.9	-13.0
	4 „ „ ..	-34.0	-17.4	-13.0

TABLE XI—*concl'd.*

Strain.	Treatment.	CONCENTRATION OF NaCl.		
		0.0093 N.	0.075 N.	0.15 N.
505	Unheated ..	-26.0	-7.8	-1.6
	15 min. 100°C.	-33.8	-17.8	-13.6
	1 hr. " "	-29.0	-14.9	-12.0
	2 hrs. " "	-32.5	-16.9	-13.0
	4 " " "	-31.9	-17.0	-13.2
W 880	Unheated ..	-24.2	-11.0	-3.4
	15 min. 100°C.	-36.0	-20.0	-16.1
	1 hr. " "	-32.5	-16.0	-15.6
	2 hrs. " "	-35.1	-19.5	-15.3
	4 " " "	-35.1	-18.2	-14.3
1617	Unheated ..	-22.1	-6.5	-2.8
	2 hrs. 100°C.	-30.6	-16.3	-12.2

There are two main points of interest in Table XI. In the first place it is evident that heating at 100°C. is only slightly more effective in raising the *P. D.* than heating at 60°C. Whatever the underlying chemical changes may be, they occur almost to the same extent at the lower temperature. Secondly, the maximum effect of heating at 100°C. is manifested during the first 15 minutes. Continued heating up to four hours does not increase the potential, and the differences noted in the table are probably within the range of observational error in the method. In every case at the salt concentration of 0.075 N the increase in potential brings the organism well outside of the critical zone where agglutination occurs in the presence of specific sera.

The marked rise in potential at the 15-minute period may explain the observation of Gardner and Venkatraman (*loc. cit.*) that 'a few minutes' exposure to 95°C. to 100°C. is enough to remove the "H" agglutinability of a (vibrio) suspension'. In physical terms this statement means that heating the vibrios raises their surface potentials almost at once, and hence increases the repulsion between the similarly charged bacterial particles. They are accordingly less agglutinable and this change is attributed to loss of 'H' antigen. This point, and its connection with the chemical changes noted in the first part of the paper, will be considered in the 'Discussion'.

That the strain we worked with, Rangoon Smooth, actually consists of 'O' antigen after it has been heated for two hours in the boiling water-bath has been shown by serological experiments that will be reported elsewhere.



## DISCUSSION.

In the data presented in this paper it is possible to distinguish two types of change in the vibrios. First are those that progress over the entire period of heating, and second those which occur within the first 15 minutes. Among the progressive changes may be noted the gradual loss of total nitrogen and amide nitrogen and the increase in humin nitrogen undergone by the residue fraction. These appear to be the result of a continuous mild hydrolysis of the vibrio. A second continuous alteration is represented by the gradual solution of A fraction in the hot water. All of these changes must disturb the reactivity of the vibrio with its homologous immune serum, both by altering the structure and by removing the highly reactive A fraction. There is also a suggestion that the structure of A which remains in the vibrio may be slightly altered.

A third progressive change occurs in the polysaccharide, which is gradually removed from the vibrio to the supernatant fluid. At the same time protein is also gradually dissolved out of the vibrio (Table V). The figures for Hausmann's analyses of the residue (Table II) and the supernatant fluid (Table VI) represent two views of the same process of progressive change in the vibrio protein. Finally, Table VIII indicates how the percentage losses of polysaccharide gradually increase with heating.

It is evident that all of the changes of this type would bring about the gradual appearance of a new structural pattern in the heated vibrios, and that the injection of this new pattern would give rise to an anti-serum differing in its range of reactivity from that against the intact organism.

The second type of change which we have found in heating the vibrios occurs in the first 15 minutes. At this period the amino-nitrogen is increased in the supernatant fluids (Table IV) and the surface potential is also increased. Further heating even to four hours does not bring about any increases comparable to those occurring at once.

All of the changes, whether gradual or rapid, are in the nature of the case connected, since they occur in and on the same vibrio. Why some occur slowly and some rapidly is a question which cannot yet be answered.

The data suggest that the destruction of 'H' antigen by heat is a complex phenomenon involving first a rapid change in the surface of the organism which is reflected in the removal of amino-nitrogen and in increased surface potential, and second by a series of slower and more progressive changes which alter the structure of the vibrio but do not further affect its surface potential. The first change destroys the 'H' antigen in the sense that the heated organisms with their higher surface charges have less or no agglutinability. The second series of changes destroys the 'H' antigen in the sense that the chemical structure is so altered that anti-serum against the heated vibrios has now a new specificity and a new range of reactivity. This latter change shows no sign of ending even after four hours' heating, and would presumably continue until the entire vibrio was in solution.

The amount of change which occurs in the destruction of 'H' antigen is shown by the following figures taken from the tables: 10 per cent of the total nitrogen, 12 per cent of the amino-nitrogen, between 10 per cent and 13 per cent of the total substance of the organisms, and from 45 per cent to 65 per cent of the polysaccharide

must be removed before the 'H' antigen disappears. It is difficult to suppose that all of this material is present in the single vibrio flagellum, and it appears probable that changes occur in the vibrio body as well.

It is also worth noting that while the amounts of material extracted from the vibrios in two hours are as shown above, all of this extract as well as the partially disintegrated vibrio has been used for injection into rabbits. In the work of Gardner and Venkatraman (*loc. cit.*), for example, the vibrios plus the extracts are injected, and the anti-sera are formed not only against the boiled vibrios, but also against any of the extracted material which has molecules large enough to cause anti-body formation.

#### SUMMARY.

Physical and chemical changes brought about in a vibrio strain by heat have been studied and the data correlated as far as possible with the double antigen hypothesis. The events following on heating are shown to be complex and both rapid and progressive alterations have been observed. The immediate effect of heat is to raise the surface potential and this change is correlated with the appearance of amino-nitrogen in the supernatant fluid. The long range effect of heat is to bring about a mild hydrolysis of the protein and the progressive removal of the polysaccharide from the vibrios. Both of these effects are concerned in bringing about the complex phenomenon known as destruction of the 'H' antigen.

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# A STATISTICAL AND BACTERIOLOGICAL ANALYSIS OF A CHOLERA EPIDEMIC IN MANIPUR STATE, ASSAM.

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## I. DESCRIPTION OF THE MANIPUR VALLEY.

MANIPUR STATE consists of a valley about 60 miles long and 25 miles broad, which is surrounded by hills. The mean elevation of the valley is approximately 2,500 feet above sea-level.

All the principal villages are situated on the banks of five rivers which flow through the valley from north to south, three of which leave the southern boundary of the State by a common outlet, the Manipur River, a tributary of the Chindwin River in Burma. These rivers are :—

- (a) The *Nambul*—arising in the foothills in the north-west, and emptying into the Logtak Lake.
- (b) The *Imphal* (the most important) having its source in the hills due north.
- (c) The *Iril*—a tributary of the Imphal River rising in the N. N. E.
- (d) The *Thoubal*—an important river rising in the north-east and, after draining the north-eastern part of the valley, joining the Imphal River 15 miles below the Capital.
- (e) The *Nambol*—arising in the W. N. W. and flowing into the Logtak Lake.

The courses of the Nambul, Imphal and Iril Rivers converge near Imphal where the three river beds are all within two miles of each other ; in fact the Nambul and Imphal are separated at one point by a distance of less than half a mile (*see Map*).

*Imphal*, the Capital, with its suburbs has a population of nearly 100,000 and is situated on the banks of the Imphal and Nambul Rivers.

The greater part of the water-supply of Imphal, which is filtered and chlorinated, is delivered to street hydrants from a distance of about 12 miles from the hills in the north-west. This supply is inadequate owing to the recent increase in the population, hence some of the inhabitants augment their requirements by utilizing the ordinary river water for domestic purposes.

## II. PREVIOUS EPIDEMICS OF CHOLERA.

A widespread epidemic occurred in 1908, which took a heavy toll of life, approximately 25,000 deaths having been reported. No further details of this epidemic are available.

Another widespread epidemic of cholera occurred between May and early September 1924. According to Police reports 8,699 persons were attacked, of whom 7,327 were reported to have died. The epidemic started with isolated sporadic cases in April, and these were reported to have been introduced from the plains of Assam via the lines of communication. The general outbreak began about the 15th May. Vital statistics for Manipur State were not kept at that time (Annual Public Health Report, Assam, 1924). It was suggested then that the Police reports for 1924 did not represent the actual number of persons affected and many cases were probably missed. The Civil Surgeon in his report on this epidemic stated : ' It does not appear to have followed any special watercourse or highway of communication ', also, ' The epidemic was associated with diminished rainfall and scarcity of water '.

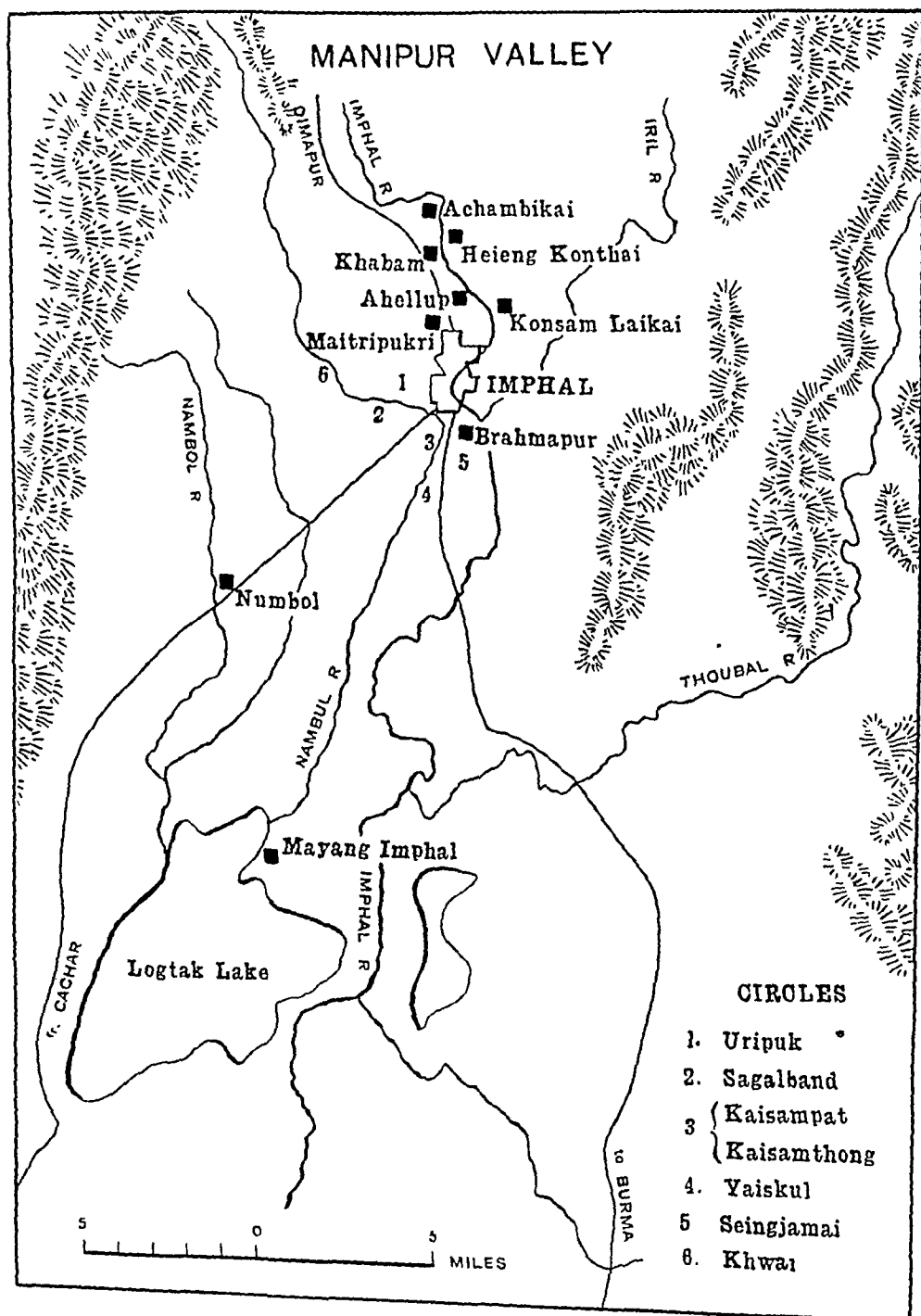
## III. THE PRESENT EPIDEMIC.

The origin of the present epidemic could not be traced to any single individual case or group of cases.

Until May of that year the valley had been free from sporadic or epidemic cholera. Early in the year a large number of Manipuris had travelled to Bengal in connection with the *Ardhodaya* festival and these are reported to have returned to Manipur towards the end of April.

About November of every year there is an exodus of Manipuri labour to the plains districts of Assam for the purpose of obtaining cold weather employment. These labourers proceed principally to Lakhimpur, Sibsagar and Kamrup Districts and usually return to their homes in April. Epidemic cholera is usually present during these months in Bengal and in Kamrup. The origin of this epidemic might therefore be attributable to one or other of these factors, as sporadic cases were first discovered early in May, coinciding with the return of these travellers. Sporadic cases subsequent to May continued to be reported until July when epidemic cholera made its appearance.

MAP.



Various factors subsequently helped to spread the epidemic.

From the 2nd to 13th July, the *Ratha Yatra* festivals were held. These festivals (known as *Khechuree* festivals) entailed a succession of communal feasts where rice cooked some hours previously was consumed liberally. Previous to this, only sporadic infection was present but immediately after this festival the first crop of epidemic cases, numbering 26, was reported. A general flare up occurred the following week with 243 reported cases. These figures do not represent the total number of cases, as it was found while collecting data that some cases had not been reported, and judging by the numbers being cremated on the banks of the rivers it would be safe to assume that the Police death reports were very conservative.

Furthermore, early in the epidemic the *Shradh* ceremony with the customary religious feasting was regularly performed on the 7th and 13th day after death. This factor added further fuel to the spread of the infection and the next week (i.e., week ending the 10th August) the epidemic was at its height with 554 cases reported. At this time the feasting associated with the *Shradh* ceremony was curtailed.

### *Rainfall.*

In 1935, until the end of July, rainfall was scanty and in consequence the river levels were low.

The rainfall for the last four years in July and August was as follows :—

Year.	July.	August.
1932 ..	12·76 inches	5·63 inches.
1933 ..	5·88 „	7·95 „
1934 ..	11·14 „	4·86 „
1935 ..	4·45 „	10·24 „

The low river levels must have been an important factor in the spread of the epidemic since the rivers were the villagers' only source of water-supply.

All the rivers, on the banks of which about 90 per cent of the population live, were ultimately infected, and it was noted that the disease was prone to spread along these watercourses.

Compared with the general sanitary condition of Indian villages in the plains of Assam, the Manipur villages are comparatively clean and tidy. Pit latrines, which are uncommon elsewhere, are to be found in all villages. However, the river banks were still used as latrines by many. During the epidemic, faecally contaminated utensils and clothing were washed in the river and the excreta of infected persons were, in many cases, thrown on to the ground and vegetable gardens in close proximity to the houses.

Between the 3rd and 16th August 8·82 inches of rain were recorded and the rivers were in high flood. This might have been one of the factors responsible for the beginning of the decline of the epidemic after that date.

#### IV. INOCULATION.

Mass inoculation was attempted from 8th August onwards, from when 80,000 inoculations were performed amongst a population of 290,000. It is not known how many of the total population were really at risk but, for the greater part, inoculations were given to those in the actually infected areas.\*

#### V. TREATMENT.

Combined cholera and dysentery bacteriophage was used for some sporadic cases in the early part of, and to a great extent, during the main epidemic.

#### GRAPH.

*Showing cases treated by different methods from 5th May to 7th September.*

##### MANIPUR CHOLERA EPIDEMIC 1935

###### TREATED WITH ESSENTIAL OILS

42 Recoveries □

34 Deaths ■

Mortality 36.36 per cent

###### TREATED WITH BACTERIOPHAGE

226 Recoveries □

106 Deaths ■

Mortality 32.33 per cent

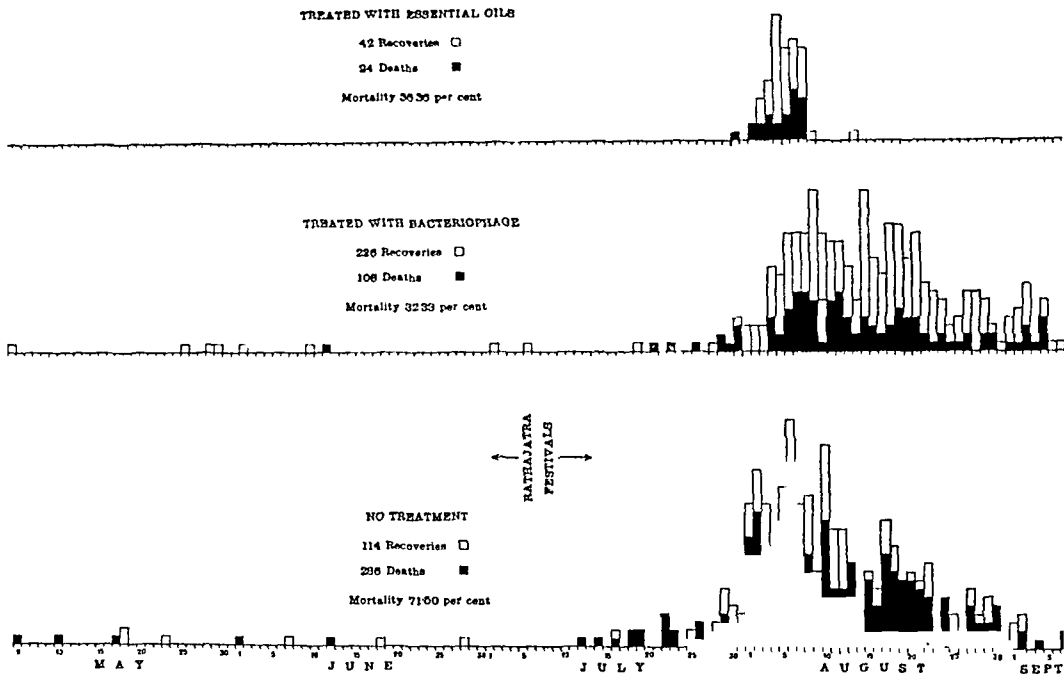
###### NO TREATMENT

114 Recoveries □

236 Deaths ■

Mortality 71.00 per cent

← RATHAJTRA  
FESTIVALS →



In areas where bacteriophage was not readily available at first, the essential oils mixture was given. This treatment was discontinued after the 9th August when bacteriophage was made freely available everywhere (*see Graph*).

Some of the people resorted to indigenous treatment by Kavirajs.†

\* The vaccine used was made at the Pasteur Institute, Shillong, had 8,000 million organisms per c.c. and was given as a single injection of one c.c.

† *Kaviraj*, locally known as *Maiba*, is a person who practises an indigenous system of medicine.



## Part I.

### STATISTICAL.

#### COLLECTION OF DATA.

During August 1935, the epidemic was confined mainly to Imphal and suburbs, and to a few villages to the north and south of this area. Subsequently the epidemic extended further to the south and to groups of villages on the east and west along the foothills. Data were collected from the town and suburb areas, and from the villages to the north and south, but not from the foothills. The data are complete as regards cases, and no village was left without being fully investigated. The data of 812 cases were obtained. These investigations required three weeks of full working days. The collection of data was facilitated as the Manipur State had placed a car at the disposal of the staff and the villages were easily accessible by good roads. In addition seven doctors and four compounders, who had treated the cases in their respective villages, accompanied one of us (N. K. G.) in his visits to these villages.

The Manipur village consists of groups of 6 to 10 small houses, around a common courtyard, and four or five of such groups formed a village. Thus by visiting one courtyard, complete data could easily be collected from 6 to 10 families. These families were found to be largely inter-related, and because of the grouping of houses as described above, there was little possibility of cases being missed when each family was interrogated.

It must be emphasized that data were collected immediately the epidemic had subsided in these areas, and while the details were fresh in the minds of the villagers.

#### GENERAL STATISTICAL ANALYSIS.

In this epidemic we are dealing with a population which is homogeneous from the standpoint of caste and customs.

The data collected, which included those from parts affected in July, August and September, give the particulars in connection with 812 cases; of these some received no treatment and others received treatment with bacteriophage or essential oils. The general mortality irrespective of treatment was 52·22 per cent. Analysing this further we find among 400 cases falling in the 'no treatment' group there were 286 deaths, i.e., a mortality rate of 71·5 per cent. Among 279 cases which received bacteriophage treatment only, 93 died, i.e., a mortality rate of 33·33 per cent, while of 55 cases receiving bacteriophage and vaccine, 15 died, a mortality of 27·27 per cent. Those receiving essential oils treatment numbered 66, of whom 24 died, i.e., a mortality rate of 36·36 per cent. Of the balance, 12 in number, who had received no treatment but had been inoculated and of whom 9 developed symptoms on the day of inoculation, 6 died and 6 recovered.

It would appear from the above, that treatment either by bacteriophage or essential oils reduced the mortality by approximately 50 per cent as compared to the no treatment group, and that among those who had previously received vaccine and were subsequently treated with bacteriophage the mortality was still further reduced.

As had been noted in the analysis of previous epidemics (Morison, 1934). no significant effect upon the mortality by 'phage administration was noticeable when 'phage was given 48 hours after the onset of the disease. This is shown in Table I :—

TABLE I.

Day of attack when 'phage given.	Total deaths.	Total recoveries.	Percentage mortality.
1	81	188	30.1
2	12	20	37.5
3	9	5	} 45.5
4	1	7	
5	5	2	
6	0	2	
7	0	2	
TOTALS ..	108	226	32.3

It is noticed that mortality among those receiving 'phage on the 1st day was 30.1 per cent, 2nd day—37.5 per cent and in the remaining group from the 3rd to 7th day—45.5 per cent.

#### INOCULATION.

No attempt has been made to evaluate the effects of inoculation with cholera vaccine, but the available data in this respect, are presented.

Up to the 8th August, cholera vaccine was used only for contacts, but after that date mass inoculation was attempted. There was some reluctance shown to inoculation and only 80,000 inoculations were performed among the population of 290,000. However, this must have had some effect in checking the spread of the disease for among the 812 cases of which we present data, only 67 had been previously inoculated. These developed cholera after inoculation as follows :—

Same day	..	..	..	..	24
2nd day	..	..	..	..	15
3rd day	..	..	..	..	8
4th day	..	..	..	..	6
5th day	..	..	..	..	5
6th day	..	..	..	..	4
8th day	..	..	..	..	3
10th day	..	..	..	..	1
14th day	..	..	..	..	1

It will thus be seen that out of the 67 cases, only 5 developed the disease after the 7th day after inoculation, i.e., the time necessary for inoculation to be effective.

## DETAILED STATISTICAL ANALYSIS.

We have analysed the available data from the following aspects in order to determine whether any of these factors would influence adversely or otherwise the results given in the general summary above:—

- (1) Was there any significant difference in the distribution of age and sex among cases falling in each of the three groups?
- (2) Was the reduction in mortality in the treatment groups only apparent because the fulminating cases were all included in the no treatment group, and died before any treatment could be made available to them?
- (3) Was the low mortality among the bacteriophage and essential oils groups due to a psychological factor which might operate with any form of treatment?
- (4) When all forms of treatment were available, did the population favour either one or the other?
- (5) Between 31st July and 9th August, 1935, inclusive, when essential oils and bacteriophage were both being used, were the results obtained by each method of treatment significant when compared with the untreated group of the same period?
- (6) Do the cases analysed represent the total case incidence of the area investigated and, if not, to what extent would 'missed cases', presumably untreated, influence the mortality rate of that group?

- (1) Was there any significant difference in the distribution of age and sex among cases falling in each of the three groups?

In Tables II, III and IV, the data for age and sex are given:—

TABLE II.

*No treatment group—age and sex.*

Age groups.	MALES.			FEMALES.			TOTALS.		
	R	D	M	R	D	M	R	D	M
1-4	10	29	74.4	8	41	83.7	18	70	79.5
5-9	17	25	59.5	13	23	63.9	30	48	61.5
10-19	12	25	67.6	13	26	66.7	25	51	67.1
20-29	6	20	76.9	17	33	66.0	23	53	69.7
30-39	5	18	78.3	4	11	73.3	9	29	76.3
40-49	3	10	76.9	2	7	77.8	5	17	77.3
50 and over	2	12	85.7	2	6	75.0	4	18	81.8
TOTALS.	55	139	71.6	59	147	71.4	114	286	71.5

R = Recoveries. D = Deaths. M = Percentage mortality.

In Table II, i.e., the no treatment group, the total mortality among 194 males was 71·6 per cent, and that among 206 females 71·4 per cent, and of the total 400 cases, 71·5 per cent. There was thus no selection in this group as regards sex. In considering the age groups, there is little evidence of any selection in any age group, with the possible exception of the group—5 to 9 years. The reduction of mortality in this group is not such as to appear of any significance.

TABLE III.

*Bacteriophage group—age and sex.*

Age groups.	MALES.			FEMALES.			TOTALS.		
	R	D	M	R	D	M	R	D	M
1-4 ..	19	6	24·0	12	9	42·9	31	15	32·6
5-9 ..	30	9	23·1	20	6	23·1	50	15	23·1
10-19 ..	28	9	24·3	18	11	37·9	46	20	29·9
20-29 ..	16	13	44·8	31	14	31·1	47	27	36·5
30-39 ..	13	8	38·1	15	10	40·0	28	18	39·1
40-49 ..	9	1	10·0	7	2	22·2	16	3	15·8
50 and over ..	6	7	53·8	2	3	60·0	8	10	55·6
TOTALS ..	121	53	30·5	105	55	34·4	226	108	32·3

R = Recoveries. D = Deaths. M = Percentage mortality.

From Table III, i.e., the bacteriophage group, it will be seen that there is no significant selection as regards sex. Regarding the age groups, those between the ages of 5 to 9 show a similar reduction of mortality as was noted in the no treatment group. The only other difference noted was among those of 40 to 49 years of age, with a mortality rate of 10 per cent in males and 22 per cent in females, but here the numbers are too small to be of real significance.

TABLE IV.

*Essential oils—age and sex.*

Age groups.	MALES.			FEMALES.			TOTALS.		
	R	D	M	R	D	M	R	D	M
1-4 ..	5	1	16.7	1	2	66.7	6	3	33.3
5-9 ..	7	1	12.5	5	3	37.5	12	4	25.0
10-19 ..	3	3	50.0	6	2	25.0	9	5	35.7
20-29 ..	1	3	75.0	6	5	45.5	7	8	53.3
30-39 ..	2	3	60.0	5	0	..	7	3	30.0
40-49 ..	0	1	..	1	0	..	1	1	..
50 and over ..	0	0	..	0	0	..	0	0	..
TOTALS ..	18	12	40.0	24	12	33.3	42	24	36.4

R = Recoveries. D = Deaths. M = Percentage mortality.

Similarly in Table IV, i.e., the essential oils group, there appears no significant selection as regards sex and the numbers in each age group are so small that no definite conclusion can be drawn from them.

The cases recorded in Tables II and III have been further distributed according to age and sex for the two groups, bacteriophage and no treatment groups. Essential oils group is not considered here, as figures under each age group are too small for such a comparison. The details are given in Table IV-A :—

TABLE IV-A.

*Distribution of cases according to age and sex for the two groups, bacteriophage and no treatment.*

Age groups.	MALES.		FEMALES.	
	No treatment.	Bacteriophage treatment.	No treatment.	Bacteriophage treatment.
1-4 ..	39	25	49	21
5-9 ..	42	39	36	26
10-19 ..	37	37	39	29
20-29 ..	26	29	50	45
30-39 ..	23	21	15	25
40-49 ..	13	10	9	9
50 and over ..	14	13	8	5

$n = 6$ ;  $X^2 = 2.777$ ;  $P$  lies between 0.9 and 0.8.       $n = 6$ ;  $X^2 = 12.147$ ;  $P$  lies between 0.1 and 0.05.

The probabilities of the differences between the distributions in the case of both sexes are greater than 0.05, which is usually taken as the level of significance. Therefore, even if the age and sex of the persons attacked influenced cholera mortality, there is no reason to believe that these factors would vitiate the comparisons of mortality rates in Table V :—

TABLE V.

*Mortality rates by periods.*

Period.	NO TREATMENT.			BACTERIOPHAGE TREATMENT.			ESSENTIAL OILS TREATMENT.		
	R	D	M	R	D	M	R	D	M
5th May to 31st July, sporadic cases.	15	35	70.0	12	10	45.5	0	1	..
1st to 7th August ..	35	98	73.7	36	17	32.1	34	18	34.6
8th to 15th August ..	36	65	64.4	69	38	35.5	8	4	33.3
16th to 23rd August ..	15	56	78.9	67	23	25.6	..	..	..
24th to 31st August ..	8	27	77.1	26	10	27.8	..	1	..
1st to 7th September ..	5	5	50.0	16	10	38.5	..	..	..
TOTALS ..	114	286	71.5	226	108	32.3	42	24	36.4

R = Recoveries. D = Deaths. M = Percentage mortality.

In Table V the mortality rates by periods are given. In the no treatment group there is no significant reduction in mortality, period by period, throughout that portion of the epidemic examined. Similarly, in the 'phage treatment group, there is little evidence to suggest that any period had a differential influence upon the efficacy of treatment.

\*This question has been further investigated by working out the  $X^2$  for the figures of each period of cholera prevalence. The method followed was that described by Fisher ('Statistical Methods for Research Workers', 5th Edition). The results are given in Table V-A :—

TABLE V-A.

Period.	Value of $X^2$ ; $n = 1$	Significance ( $P = 0.05$ ).
5th May to 31st July ..	3.9273	Significant.
1st to 7th August ..	27.8005	Significant.
8th to 15th August ..	17.2904	Significant.
16th to 23rd August ..	45.1469	Significant.
24th to 31st August ..	17.3296	Significant.
1st to 7th September ..	..	Not significant $P = 0.8127$

When the separate values of  $X^2$  are summed, we get a quantity greater than 110, and for the corresponding sum of degrees of freedom, namely 6, this value of  $X^2$  is definitely significant. Hence, the conclusion is that bacteriophage treatment has brought down mortality.

The essential oils treatment was only given during the period from 1st to 9th August, i.e., at the beginning of the epidemic, and is thus not comparable to the other groups from the standpoint of periods. We shall refer to this aspect again later.

- (2) Was the reduction in mortality in the treatment groups only apparent because the fulminating cases were all included in the no treatment group, and died before any treatment could be made available to them ?

\*Acknowledgment.—We are indebted to Dr. K. C. K. E. Raja of the All-India Institute of Hygiene, Calcutta, for working out Tables IV-A and V-A, and comments thereon.

In this connection we accept the reasoning put forward by Morison (*loc. cit.*) and have presented our data in Table VI in the same manner :—

TABLE VI.

	No treatment.	Bacteriophage within 24 hours of onset.	Bacteriophage between 24 and 48 hours of onset.	Essential oils.
Recoveries ..	114	188	20	42
Total deaths ..	286	81	12	24
Less deaths within 24 hours of onset.	175	..	..	..
		(43 not de- ducted).	(1 not de- ducted).	(14 not de- ducted).
Nett deaths ..	111	81	12	24
Percentage mortality ..	49.3	30.1	37.5	36.4

Among the 286 deaths in the no treatment group, 175 or 59.4 per cent died within 24 hours of the onset of the disease. These were probably fulminating cases to whom any treatment may not have been available. Among 269 cases who received bacteriophage on the 1st day of illness, 81 died from 1 to 11 days later, and 43 of these died on the day of onset. The latter were presumably fulminating cases too and comparable to those who died on the 1st day in the untreated group.

If now we deduct these 175 deaths from the total deaths of the no treatment group, we would have 225 cases with 111 deaths, i.e., a mortality rate of 49.3 per cent. Even if we do not deduct the 43 deaths occurring on the 1st day in the bacteriophage group the mortality rate, when bacteriophage was administered on the 1st day, was 30.1 per cent and similarly when bacteriophage was given on the 2nd day, the mortality rate was 37.5 per cent.

Among the essential oils group of 66 cases with 24 deaths, 14 died within the first 24 hours. These latter, similar to the bacteriophage group, were presumably fulminating cases. Without deducting these from the total deaths the mortality rate is 36.4 per cent.

It will be seen that after thus abstracting no treatment deaths within 24 hours, the data presented will certainly not be weighted in favour of bacteriophage or essential oils.

- (3) Was the low mortality among the bacteriophage and essential oils groups due to a psychological factor which might operate with any form of treatment?

We have, up to this point, presented our data in accordance with the method of analysis previously adopted by Morison (*loc. cit.*). In the previous epidemics which were analysed, the no treatment group was comprised of cases which had not



received treatment in any form. In the Manipur epidemic, however, among the 400 cases which we have so far considered as 'no treatment cases', 223 had received treatment by 'Kavirajs', i.e., by those who were using an indigenous method of treatment. The reason for not excluding these cases in the beginning were two-fold : to present data comparable with that of former epidemics, when it is possible that such indigenous treatment data may not have been obtained ; and secondly, absence of precise knowledge regarding either the nature or the homogeneity of this form of treatment. It is possible that such form of treatment had some beneficial effects either medicinally or from the psychological view-point. To elicit these factors we have further analysed the so-called no treatment group in Table VII :—

TABLE VII.

Day of death.	NO TREATMENT.		' KAVIRAJI ' TREATMENT.	
	Recoveries.	Deaths.	Recoveries.	Deaths.
1	..	103	..	72
2	..	26	..	38
3	..	16	..	8
4	..	4	..	6
5	..	..	..	2
6	..	..	..	6
7	..	..	..	3
8	..	..	..	..
9	..	..	..	..
10	..	1	..	1
	27	..	87	..
TOTALS ..	27	150	87	136
Percentage mortality.	84·7		61·0	

It will be seen from this grouping that of 177 cases which received no treatment there were 150 deaths, i.e., a mortality rate of 84·7 per cent and in the Kaviraji treatment of 223 cases with 136 deaths, the mortality rate is reduced to 61·0 per cent. This reduction of nearly 24 per cent in the mortality can be attributed to three possible factors, viz. :—

1. Selection of cases.
2. Efficacy of Kaviraji treatment, and
3. A possible psychological effect of some form of treatment.

It has previously been suggested that the reduction of mortality among those treated with bacteriophage might have been due to factors 1 and 3 cited above.

In Table VII, where data for Kaviraji treatment are presented, it would appear that, while there was some sort of selection of cases receiving that treatment, they

had lived sufficiently long to receive some form of treatment and at least 72 out of 223 cases did receive treatment on the 1st day of disease. The reduction in mortality to 32·3 per cent in the bacteriophage group therefore would appear significant, because if selection, and the psychological effects of any form of treatment were the only other factors, we should have obtained a mortality rate in the bacteriophage treated group similar to that in the Kaviraji treatment group, viz., 61·0 per cent in this case.

- (4) When all forms of treatment were available did the population favour either one or the other ?

TABLE VIII.

Area.	NO TREATMENT.		KAVIRAJI TREATMENT.		BACTERIOPHAGE.		ESSENTIAL OILS.	
	R	D	R	D	R	D	R	D
<i>Town group.</i> —								
Uripuk .. ..	3	12	4	1	32	17	5	3
Sagalband .. ..	0	7	5	2	20	18	0	2
Kaisambat .. ..	1	15	4	1	36	14	0	0
Yaiskul .. ..	1	1	1	3	15	6	0	0
Seinjamai .. ..	5	11	15	5	27	15	0	0
Khwai .. ..	5	17	2	3	15	5	14	7
Brahmapur .. ..	3	18	0	0	13	7	0	0
Miscellaneous ..	0	2	2	4	2	4	0	1
TOTALS ..	18	83	33	19	169	86	19	13
Percentage mortality ..	82·2		36·5		33·7		40·6	
<i>Rural group.</i> —								
Mantripulchri ..	0	14	4	19	11	3	0	0
Khalam Ahellup ..	1	0	30	28	2	3	20	11
Haieng Konthai ..	1	20	4	31	4	4	3	0
Achambikai ..	0	0	6	26	3	0	0	0
Konsumlaikai ..	2	8	3	2	2	2	0	0
Numbol .. ..	3	9	0	2	24	5	0	0
Mayang Imphal ..	1	8	0	0	3	1	0	0
Miscellaneous ..	1	8	7	9	8	4	0	0
TOTALS ..	9	67	54	117	57	22	23	11
Percentage mortality ..	88·2		68·4		27·8		32·4	
GRAND TOTALS ..	27	150	87	136	226	108	42	24
GRAND PERCENTAGE MORTALITY.	84·7		61·0		32·3		36·4	

R = Recoveries.

D = Deaths.

In Table VIII, we have presented the data of cases in two groups: (a) those cases occurring in Imphal Town or within a radius of three miles and (b) those cases occurring at a further distance from the town.

These cases are distributed in Table IX according to the respective treatment which they received:—

TABLE IX.

Treatment.	TOWN GROUP (440 CASES).		RURAL GROUP (360 CASES).	
	Number.	Percentage.	Number.	Percentage.
No treatment ..	101	23.0	76	21.1
Kaviraji ..	52	11.8	171	47.5
Bacteriophage ..	255	58.0	79	21.9
Essential oils ..	32	7.2	34	9.5

It will be seen from Table IX that in the town group, where presumably all three forms of treatment were easily available, that the population preferred bacteriophage treatment to Kaviraji treatment, 58 per cent of the total cases having received the former treatment, compared to 11.8 per cent of the latter.

With respect to essential oils they were only used between the 30th July and 9th August principally in the Khwai and Khalam Ahellup Circles before bacteriophage was freely available..

In the rural group 47.5 per cent of the total cases received Kaviraji treatment and only 21.9 per cent received bacteriophage. • It would appear that in the rural areas bacteriophage was not so freely available, and so the population resorted to the only form of treatment which they could get, i.e., the Kaviraji treatment. This statement is borne out by the fact that in Numbol, which is on the Cachar road and readily accessible to the town, 29 cases out of total 43 cases received bacteriophage. Similarly, in Mantripulchri, we found that Kaviraji treatment was used extensively from the 1st to 15th August, when it was practically entirely replaced by the use of bacteriophage.

It would appear, therefore, that the people had confidence in the treatment made available by Government agencies, and took that treatment readily. If there was any preference for treatment it was for the bacteriophage as soon as it was made available.

In Table VIII, when comparing the town and rural areas according to the no treatment, bacteriophage and essential oils groups it will be seen that the respective

mortalities under these headings in both areas were significantly close to the mean mortality rate for each respective group. In the Kaviraji treatment, however, the mortality rate in the rural area (68·4 per cent) is considerably higher than that of the town group, viz., 36·5 per cent. This reduction in mortality in the latter is indeed comparable to that obtained either by 'phage or essential oils. We are unable to explain this difference in the Kaviraji treatment mortality rates in the two areas in the absence of definite knowledge of the general nature of that treatment\*.

- (5) Between 31st July and 9th August, 1935, inclusive, when essential oils and bacteriophage were both being used, were the results obtained by each method of treatment significant when compared with the untreated group of the same period ?

TABLE X.

*Analysis of cases occurring between 31st July and 9th August.*

	No treatment.	Kaviraji.	Bacteriophage.	Essential oils.
Recoveries ..	9	37	56	42
Deaths ..	61	58	33	24
TOTALS ..	70	95	89	66
Percentage mortality.	87·1	61·1	36·4	37·1

It will be seen from Table X, that the mortality rates in the bacteriophage and essential oils groups would appear to be significantly lower than those of the Kaviraji or 'no treatment' groups. It will be seen also that the total cases in all the four groups are comparable in numbers—a significant point in this table.

- (6) Do the cases analysed represent the total case incidence in the area investigated and, if not, to what extent would 'missed cases', presumably untreated, influence the mortality rate of that group ?

The above data were collected by one of us (N. K. G.), who visited every affected house in the area investigated. Other houses were also visited, to obtain data

\* It may be interesting to note that in the course of the investigation, it was found that a Kaviraj was treating cases with the clear supernatant fluid obtained from a stool of a cholera case, and another Kaviraj had used this treatment during an epidemic in 1924 for the treatment of his son.

should a mild case not have been reported, but every inmate was not questioned. Early cases in May and June, not previously reported, as well as some unreported cases occurring during the epidemic period, were thus found by this procedure. However, it is possible that some milder cases might yet have remained undetected. If so, these cases were obviously untreated cases, and would thus fall into the 'no treatment' group. In recognizing this factor, we have attempted to ascertain to what extent these undetected cases may have been present, in order to vitiate significantly the mortality rate shown in the 'no treatment' group.

As shown in Tables VII and VIII there were a total of 177 cases who did not receive treatment of any description. In order to reduce the mortality in this group, i.e., 84.7 per cent to 61.0 per cent, observed with the Kaviraji treatment or 32.3 per cent with bacteriophage, it would have been necessary for us to have missed 68 cases in the first instance and 288 cases in the latter.

In view of the detection of previously unreported cases, it is not likely that 68 cases could have been missed and it seems almost improbable that 288 cases, i.e., 36 per cent of the total cases recorded, could possibly have remained undetected.

#### INFECTIVITY.

We have used Morison's formula as a measure of infectivity, i.e., the index  $C$ , the ratio of  $y\sqrt{\frac{a+b}{2}}$  multiplied by 1,000, where  $a$ =primary case,  $b$ =the remaining persons in the same house, and  $y$ =the secondary cases in the same house.

We show in Table XI, the value of  $C$  for 6 group headings:—

TABLE XI.

	Combined no treat- ment and Kaviraji treatment groups.		No treat- ment.		Kaviraji treatment.		Bacterio- phage within 48 hours.		Bacterio- phage after 48 hours.		Essential oils.	
	1	2	1	2	1	2	1	2	1	2	1	2
Primary cases in a house.												
Total popula- tion.	1,509	131	709	51	800	80	1,025	55	202	0	278	20
Primary cases (a).	275	45	128	16	147	29	182	19	37	0	47	7
Population at risk (b).	1,234	86	581	35	653	51	843	36	165	0	231	13
Secondary cases (y).	191	8	50	6	51	2	38	1	22	0	12	5
Value of $C$ ..	133.9	122.1	141.0	235.3	127.5	50.0	74.1	36.4	217.8	0	80.5	500

When there was one primary case in a house the infectivity rates were as follows :—

No treatment group	..	..	..	141·0
Kaviraji treatment	..	..	..	127·5
Bacteriophage within 48 hours		..	..	74·1
Bacteriophage after 48 hours		..	..	217·8
Essential oils	..	..	..	80·5

It would appear that bacteriophage and essential oils had reduced the infectivity ratio to some extent. Bacteriophage given after 48 hours shows a higher infectivity ratio than do either the no treatment or the Kaviraji treatment groups. These cases, as they lived for 48 hours before receiving bacteriophage, were as infective as the no treatment or Kaviraji treatment groups cases or more so, since the majority of cases in the two latter groups had died within 24 hours.

On the assumption that cases surviving after 24 hours are more likely to give rise to secondary cases than those who have died within this time, we have deducted such deaths from each group, and have calculated the infectivity rates of those surviving after 24 hours according to value *C* by the same formula. The altered infectivity rates were as follows :—

No treatment	..	..	..	316·5
Kaviraji treatment	..	..	..	140·1
Bacteriophage within 48 hours		..	..	77·4
Bacteriophage after 48 hours		..	..	217·8
Essential oils	..	..	..	90·6

From these figures it would appear that the infectivity ratio is reduced with any form of treatment.

While it is possible that bacteriophage may alter the infectivity of the vibrio by its biological action, we are unable to explain the reduction in the value of *C* in the essential oils and Kaviraji treatment groups unless we assume that those who resort to any form of treatment also take hygienic precautions against infection in the presence of a primary case. Indeed field experience has shown that in certain houses more intelligence is shown than in others regarding hygienic measures.

## Part II.

### BACTERIOLOGICAL FINDINGS.

\* Immediately on receipt of the report of the epidemic, a field unit in charge of a bacteriologist was sent to Imphal to investigate the epidemic bacteriologically. The unit worked at Imphal from the 11th August to 1st September, during which period specimens of stools from 70 cases from different areas were examined and from these 37 specimens yielded vibrios which were identified as cholera vibrios by the usual laboratory tests. Some of the specimens were obtained from cases who had received no treatment, some within 24 hours, after bacteriophage was administered to them, and from some to whom bacteriophage was given at least 48 hours before the specimens were taken.

The specimens were examined within four hours of collection. A flake of mucus was selected, washed in either broth or saline, and plated direct on to a bile-salt agar plate. When flakes were not obtainable, a loopful of material was plated after preliminary enrichment in 1 per cent peptone water.

In the study of the vibrios the following sugars were used : Lactose, glucose, dulcitol, mannitol, saccharose and maltose.

For agglutination tests, the following high titre sera were used :—

1. Serum *vs.* *Vibrio* 653 Titre 1-5,000. 'OH'.
2. Serum *vs.* *Vibrio* Inaba Titre 1-1,000. 'O' only.
3. Serum *vs.* *Vibrio* Ogawa Titre 1-1,000. 'O' only.

Sera Nos. 2 and 3 were obtained from the Director, Central Research Institute, Kasauli.

The general findings are given in Tables XII, XIII and XIV :—

In Table XII particulars of eight vibrios from cases receiving no treatment are listed. All gave typical sugar reactions, and were smooth to Millon's test. The first five gave typical agglutination reactions with Inaba and 653 (Shillong) sera and differential agglutinations with Ogawa serum (Gardner and Venkatraman, 1935). These vibrios are therefore typical cholera vibrios. The remaining three strains gave an indication of a different antigenic structure, especially when tested with Inaba serum. SM 47 also shows great reduction in titre with serum 653.

In Table XIII, particulars of 23 strains isolated from cases within 24 hours of their receiving bacteriophage are given. All gave typical sugar reactions, though one fermented dulcitol in addition and all were smooth to Millon's test. Their agglutination reactions are typical with the three sera used ; the slight variations which are noticed, require no comment.

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\* The strains collected were investigated at the Pasteur Institute, Shillong, by Doctors A. C. Vardon, I.M.D., C.P.H., N. M. Maitra, B.Sc., M.B., and N. C. Roy, L.M.P., L.T.M.

TABLE XII.

*Vibrio isolated from 410 treatment cases.*

Strain No.	Date of onset.	Date specimen collected.	Patient recovered or died.	Sugar reactions.	Indol test.	Cholera red test.	Millon's test—Smooth or Rough.	Hemolysis (sheep cells).	AGGLUTINATION TESTS—SER.		
									Inaba ('O').	Ogawa ('O').	653 ('OH').
SM 12 ..	11-8-35	11-8-35	Died	Typical	++	—	S	Non-haemolytic	1,000	250	5,000
SM 29 ..	16-8-35	16-8-35	"	"	++	Tr.	S	"	1,000	250	5,000
SM 30 ..	16-8-35	17-8-35	"	"	++	Tr.	S	"	1,000	250	5,000
SM 32 ..	16-8-35	19-8-35	"	"	++	+	S	"	1,000	250	5,000
SM 37 ..	19-8-35	20-8-35	Recovered	"	++	—	S	"	1,000	500	2,500
SM 47 ..	17-8-35	23-8-35	"	"	+	—	S	"	Tr. in all dil. to 1,000.	125	Tr. in all dil. to 125.
SM 57 ..	28-8-35	29-8-35	Died	"	++	+	S	"	Tr. in all dil. to 2,500.	125	2,500
SM 65 ..	1-9-35	3-9-35	"	"	++	+	S	"	Tr. in all dil. to 2,500.	125	2,500



TABLE XIII.

*Vibrios isolated from cases receiving bacteriophage.*

(Specimens obtained within 24 hours of receiving bacteriophage.)

Strain No.	Date of onset.	Date specimen collected.	Patient recovered or died.	Sugar reactions.	Indol test.	Cholera red test.	Millon's test—Smooth or Rough.	Hæmolysis (sheep cells).	AGGLUTINATION TESTS—SERA.		
									Inaba ('O').	Ogawa ('O').	653 ('OH').
SM 2 ..	10-8-35	12-8-35	Recovered	Typical	++	++	S	Non-hemolytic	1,000	250	2,500
SM 3 ..	10-8-35	12-8-35	"	"	++	++	S	"	1,000	250	2,500
SM 4 ..	10-8-35	12-8-35	"	"	++	++	S	"	1,000	125	1,000
SM 5 ..	10-8-35	12-8-35	"	"	++	++	S	"	1,000	125	2,500
SM 6 ..	12-8-35	14-8-35	"	"	++	++	S	"	1,000	250	2,500
SM 16 ..	12-8-35	14-8-35	"	"	++	++	S	"	1,000	125	2,500
SM 17 ..	12-8-35	14-8-35	Died	"	++	++	S	"	1,000	125	2,500
SM 18 ..	12-8-35	14-8-35	"	"	++	++	S	"	1,000	125	2,500
SM 20 ..	13-8-35	15-8-35	"	"	++	++	S	"	1,000	500	5,000
SM 21 ..	14-8-35	15-8-35	Recovered	"	++	Tr.	S	"	1,000	250	5,000
SM 23 ..	14-8-35	16-8-35	Died	"	++	Tr.	S	"	1,000	250	5,000
SM 24 ..	15-8-35	16-8-35	"	"	++	Tr.	S	"	2,500	125	5,000
SM 25 ..	15-8-35	16-8-35	Recovered	"	++	Tr.	S	"	1,000	250	5,000
SM 26 ..	15-8-35	16-8-35	"	"	++	Tr.	S	"	2,500	250	5,000
SM 27 ..	18-8-35	20-8-35	"	"	++	Tr.	S	"	1,000	250	5,000
SM 28 ..	14-8-35	20-8-35	"	"	++	Tr.	S	"	1,000	125	5,000
SM 31 ..	17-8-35	20-8-35	"	"	++	+	S	"	1,000	250	2,500
SM 35 ..	18-8-35	20-8-35	"	"	++	-	S	"	1,000	500	1,000
SM 36 ..	19-8-35	20-8-35	"	Typical but dulcitate also fermented.	++	+	S	"	1,000	250	2,500
SM 38 ..	19-8-35	20-8-35	Died	Typical	++	+	S	"	1,000	250	1,000
SM 42 ..	21-8-35	22-8-35	Recovered	"	++	Tr.	S	"	1,000	250	5,000
SM 43 ..	21-8-35	22-8-35	"	"	++	Tr.	S	"	2,500	250	2,500
SM 44 ..	21-8-35	22-8-35	Died	"	++	+	S	"	500	125	2,500
SM 46 ..	22-8-35	23-8-35	Recovered	"	++	+	S	"	500	50	2,500

TABLE XIV.

*Vibrios isolated from cases receiving bacteriophage.*

(Specimens obtained after 48 hours of receiving bacteriophage.)

Strain No.	Date of onset.	Date specimen collected.	Patient recovered or died.	Sugar reactions.	Indol test.	Cholera red test.	Millon's test—Smooth or Rough.	Hemolysis (sheep cells).	AGGLUTINATION TESTS—SERA.		
									Inaba ('O').	Ogawa ('O').	653 ('OH').
SM 7 ..	9-8-35	13-8-35	Recovered	Typical	++	—	S	Non-hemolytic	1,000	125	2,500
SM 13 ..	11-8-35	14-8-35	Died	"	++	—	S	"	1,000	125	2,500
SM 33 ..	13-8-35	19-8-35	Recovered	"	++	—	S	"	Tr. in all dil. to 500.	Tr. in all dil. to 125.	Tr. in all dil. to 1,000.
SM 39 ..	15-8-35	20-9-35	"	Typical but dulcitate also fermented.	++	—	S	"	500	Tr. in all dil. to 125.	5,000
SM 40 ..	16-8-35	21-8-35	"	Typical	+	Tr.	S	"	2,500	125	500 and tr. up to 5,000
SM 45 ..	18-8-35	21-8-35	"	"	++	+	S	"	250	Tr. in all dil. to 125.	250 and tr. up to 5,000

In Table XIV particulars of six strains are given. The stools were collected in these cases at least 48 hours after they had been treated with bacteriophage. These are listed separately to ascertain whether their antigenic structure had been altered by the administration of bacteriophage. All gave typical sugar reactions except one which also fermented dulcitol. All were smooth to Millon's test. The agglutination reactions of the first two are typical. The others show variations as were noticed in Table XII, but in this case the variations were also noticeable with the Ogawa serum.

A perusal of Tables XII, XIII and XIV would suggest, that atypical agglutination reactions were obtained in all groups in vibrios isolated at the end of the epidemic. It was also found that between 23rd August and 3rd September, although 23 specimens were examined only two gave positive results, i.e., SM 57 and SM 65.

We have also investigated the lysability or resistance of strains to 'phage types, and the action of different brews of therapeutic 'phage issued for use in the field. The therapeutic brews used for these tests had the following composition :—

Brew No.	Types present.	Strength.
419	A B C D G K	$1 \times 10^{12}$
422	A B C D E F G H J K	$1 \times 10^{12}$
423	A B C D E F H J K	$1 \times 10^9$
424	A B C D E F G H J	$1 \times 10^{12}$
430	A B C D F G H J	$1 \times 10^{11}$
431	A B C E G H J	$1 \times 10^{10}$

The readings regarding lysability of cultures by these brews were taken at the end of four hours. At the end of 24 hours only minor variations were noted. The results are given in Tables XV, XVI and XVII.

TABLE XV.

*Action of cholera bacteriophages on vibrios listed in Table XII.*

(Readings taken after four hours.)

Strain No.	ACTION OF INDIVIDUAL TYPES OF CHOLERA 'PHAGES.		LYSABILITY TO THERAPEUTIC 'PHAGE BREWS.					
	Lysed by types.	Resistant to types.	419	422	423	424	430	431
SM 12 ..	BCDEFHJ	AGKL	+++	++	++±	++±	+++	+++
SM 29 ..	BCDEFHJ	AGKL	+++	++	+++	+++	+++	+++
SM 30 ..	BCDEFHJ	AGKL	++	++	++	+++	+++	+++
SM 32 ..	BCDEFHJ	AGKL	++±	++	+++	+++	+++	+++
SM 37 ..	BCDEFHJKL	AG	+++	++	++	++±	+++	+++
SM 47 ..	Nil	All types	—	—	—	—	—	—
SM 57 ..	BCDFHJ	AEGKL	*	*	*	++±	*	*
SM 65 ..	BCDEFHJ	AGKL	*	*	*	±	*	*

\* Not tested.

In this, and subsequent tables, the results of lysability tests are shown thus:—

+++ = complete lysis.  
 ++±  
 ++  
 +±  
 ±  
 +  
 — } = partial lysis and gradations.  
 — = no lysis.

TABLE XVI.

*Action of cholera bacteriophages on vibrios listed in Table XIII.*

Strain No.	ACTION OF INDIVIDUAL TYPES OF CHOLERA 'PHAGES.		LYSABILITY TO THERAPEUTIC 'PHAGE BREWS.					
	Lysed by types.	Resistant types.	419	422	423	424	430	431
SM 2 ..	C D E F G H J K L	A B	++	+++	+++	+++	+++	+++
SM 3 ..	B C D E F G H J	A K L	+++	++±	++	+++	+++	+++
SM 4 ..	B C D E F H J	A G K L	++±	++	++	+++	+++	+++
SM 5 ..	B C D E F H J	A G K L	+++	++	++	++±	+++	+++
SM 6 ..	B C D E F G H J K L	A	++	+++	+++	+++	+++	+++
SM 15 ..	B C D E F H J	A G K L	+++	++±	++	+++	+++	+++
SM 17 ..	B C D E F H J	A G K L	++±	++	±	±	+++	+++
SM 18 ..	B C D F G H J	A E K L	+++	++	++	++	+++	+++
SM 20 ..	B C D E F H J	A G K L	++±	++	±	++±	+++	+++
SM 21 ..	B C D E F H J	A G K L	--	++	++	+++	+++	+++
SM 23 ..	B C D E F H J	A G K L	+++	+++	+++	+++	+++	+++
SM 24 ..	B C D F G H J	A E K L	+++	+++	+++	+++	+++	+++
SM 25 ..	B C D F G H J	A E K L	+++	+++	+++	+++	+++	+++
SM 26 ..	B C D F G H J	A E K L	+++	+++	+++	+++	+++	+++
SM 27 ..	B C D E F H J	A G K L	+++	++	+++	+++	+++	+++
SM 28 ..	B C D E F H J	A G K L	+++	+++	++±	+++	+++	+++
SM 31 ..	B C D E F H J	A G K L	+++	+++	+++	+++	+++	+++
SM 35 ..	B C D E F H J	A G K L	+±	±	±	+	+++	+++
SM 36 ..	B C D E F H J	A G K L	+	+	+	±	±	±
SM 38 ..	B C D E F G H J	A K L	+++	++	+++	++	+++	+++
SM 42 ..	B C D E F G H J	A K L	++±	++±	+++	+++	+++	+++
SM 43 ..	B C D E F G H J	A K L	+++	+++	+++	+++	+++	+++
SM 44 ..	B C D E F H	A G J K L	±	++	+++	+++	+++	+++
SM 46 ..	B C D E F H	A G J K L	+++	±	±	±	+++	+++

TABLE XVII.

*Action of cholera bacteriophages on vibrios listed in Table XIV.*

Strain No.	ACTION OF INDIVIDUAL TYPES OF CHOLERA 'PHAGES.		LYSABILITY TO THERAPEUTIC 'PHAGE BREWS.					
	Lysed by types.	Resistant to types.	419	422	423	424	430	431
SM 7 ..	B C D E F H J	A G K L	++±	++	++±	++±	+++	+++
SM 13 ..	B C D E F G H J	A K L	+++	++	+++	++±	+++	+++
SM 33 ..	Nil	All types	—	—	—	—	—	—
SM 39 ..	B C D E F H J K L	A G	++	+++	+++	+++	+++	+++
SM 40 ..	B C D E F G H J	A K L	+++	++	++±	++±	+++	+++
SM 45 ..	B C D E F G H J K L	A	++±	+++	+++	+++	+++	+++

It will be seen in these tables that two strains only, viz., SM 33, SM 47, were resistant to all types of cholera bacteriophages. These strains, as might be expected, were resistant to therapeutic 'phage brews. Their agglutination reactions were also atypical, but other characters such as fermentation of sugars, etc., were typical of a cholera vibrio. Strains SM 6 and SM 45 were resistant to type 'A' 'phage only, though only the former showed typical agglutination. In the action of therapeutic 'phage on these strains, a similar difference was noted, in that brew No. 419 did not give complete lysis in four hours. Twenty strains were resistant to A, G, K and L types. The therapeutic 'phage brews show variations in lysability though their agglutination reactions of these strains were more or less typical.

Summarizing these results we find that in all the three groups, the biochemical characters of the vibrios isolated simulate one another. Those vibrios isolated in the early part of the epidemic in all instances give true agglutination reactions. Towards the end of the epidemic variations in agglutinability are noticeable.

As regards resistance to individual 'phage types, the three groups do not differ materially. There was no correlation between 'phage resistance and agglutinability. With two exceptions the therapeutic 'phage lysed all the vibrios isolated. In studying the action of different brews on the individual vibrios, variations in lysability were noticed. This is important, for as the brews may differ in their action because of the absence in them of one or more 'phage types, varying results in the use of therapeutic 'phage in the field are possible.

## SUMMARY.

- (1) A cholera epidemic in the Manipur Valley of Assam is described.
- (2) The general statistical analysis shows that the mortality in 812 cases, regardless of treatment, was 52·2 per cent, that among 400 cases receiving no treatment 71·5 per cent, among 334 cases treated with bacteriophage, 32·3 per cent, and among the 66 treated with essential oils 36·4 per cent. Of the 812 cases, 67 had been inoculated. The majority of cases are thus found to be amongst the uninoculated.
- (3) The detailed statistical analysis is given to evaluate the question of possible selection in the treated groups; any psychological factor operating amongst treated cases; whether the population favoured one or other form of treatment; and whether the cases recorded represented the total incidence of the disease in the area, and if any cases had been missed to what extent they influenced the statistical analysis.
- (4) In previous epidemics, we had no knowledge as to whether indigenous form of treatment was used during an epidemic. In this epidemic such treatment was given by Kavirajs. The influence of this factor is discussed. This factor has also facilitated the analysis noted above.
- (5) The infectivity, as evaluated by Morison's formula, is given. The ratio was lower both in the bacteriophage and essential oils groups. The influence of any form of treatment on infectivity is discussed.
- (6) The epidemic has been investigated bacteriologically and was found to be due to a typical agglutinating vibrio. Details are given of cholera vibrios obtained from those who received no treatment, and of others isolated at different periods after the cases had received bacteriophage treatment.

## ACKNOWLEDGMENTS.

Our thanks are due to the Maharaja of Manipur for placing the resources of the state at our disposal, Colonel C. E. Palmer, I.M.S., Inspector-General of Civil Hospitals, Assam, and Lieut.-Colonel T. D. Murison, I.M.S., Director of Public Health, Assam, for their assistance and to Dr. R. B. Lal and Dr. K. C. K. E. Raja of the All-India Institute of Hygiene, Calcutta, for their valuable suggestions in the preparation of this paper. We are also indebted to Mr. A. B. Smart for the preparation of the Map.

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## AN EPIDEMIC OF CHOLERA IN MONDAIR VILLAGE (HABIGANJ SUBDIVISION, ASSAM).

BY

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THE Habiganj subdivision was selected in 1932 as one of two areas in Assam, in which to determine the effects of bacteriophage in the control of cholera epidemics. The principles underlying this form of control have already been described by Morison *et al.* (1933). By the distribution of bacteriophage to each village, early treatment of cases of cholera is facilitated. An account is given here of an outbreak of cholera in a village in this area.

Mondair is a small village situated on the southern bank of the Ratna, a tributary of the Barak River and at a distance of about 10 miles from Habiganj Town. The village runs east to west for about five furlongs along the river bank. It consists of a series of huts built on small artificial mounds, about 12 in number, situated in most cases very close to each other. The total population is 751, Mohammedans predominating. The river is the only source of water-supply, excepting one tank adjacent to, and used by, one small group of huts only (*see Map*).

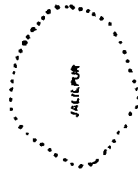
### THE ORIGIN OF THE OUTBREAK.

It was ascertained that during the night of 2nd April, a Hindu lady, a pilgrim returning from a festival in Lakhai (on the banks of the Dhalisiri River bordering on Mymensingh District), passed through this village and, crossing the river at the Ferry Ghat, proceeded towards Baniachung. She was suffering from vomiting and diarrhoea, and was so ill that she had to be carried in a 'Sowari'. At the Ferry Ghat she had to wait for about three hours before arrangements could be made for her transport. It is suspected that this case was the cause of the importation of cholera into Mondair. At this Ferry Ghat on the following day (3rd April), a woman of Mondair waited all day from the early hours of the morning, to receive her relatives who were due to arrive from Baniachung. During the night this woman developed cholera and died. This was the first case in the village and this case gave rise to an epidemic resulting in 65 attacks and 33 deaths. The last case was reported on



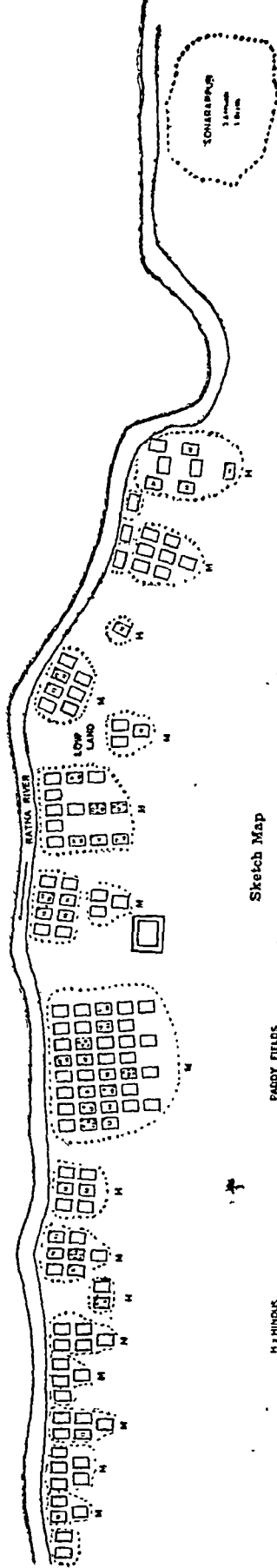
Cholera Epidemic, April 1935

# MAP



PADDY FIELDS

PADDY FIELDS



Sketch Map

## VILLAGE MONDAIR

THANA BANITACHONG  
SUB-DIVISION HABIGANJ  
DISTRICT SYLHET

PADDY FIELDS

PADDY FIELDS

H = HINDUS

M = MUHAMMADANS

Scale of 1 inch to 1 mile  
 0 1/2 1 1 1/2 2  
 Feet Miles

22nd April, 1935. The dates on which the cases occurred in different houses, and the total number of cases and deaths in each group of huts are recorded in the Map. It was ascertained that during the epidemic, i.e., between the 3rd and 22nd April, there had occurred a large number of cases of diarrhoea which had received bacteriophage but which were not recorded as cholera. Some of these appear to have been cases of cholera. The evidence in this connection will be discussed under 'Comments' at the end of this paper.

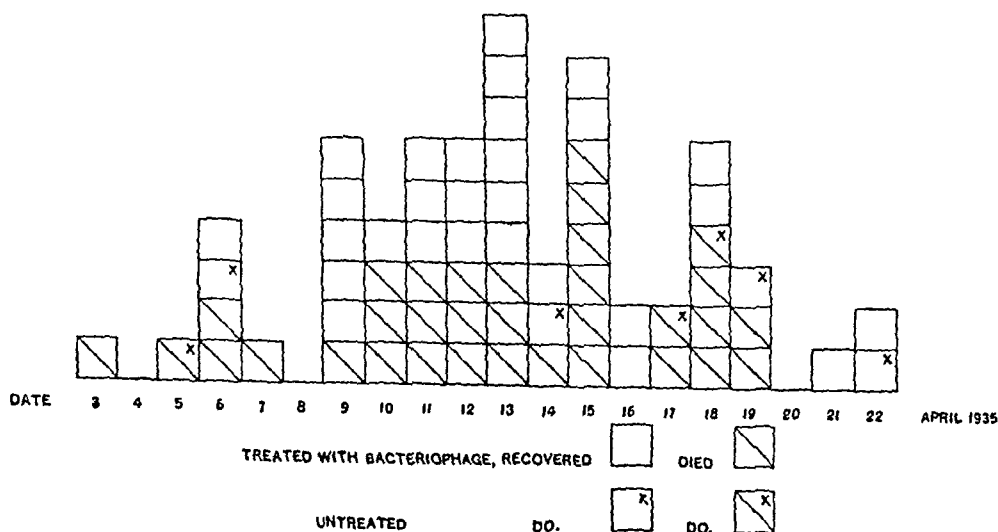
Although some early cases may have been infected through the medium of the river water, it does not appear that the bulk of the epidemic was due to the contamination of river water which was the only source of supply. While a few cases occurred in houses situated down the stream, the epidemic spread rapidly eastwards and reached Sonarampur village, about half a mile up stream from Mondair by the 14th April. Western portions of Mondair itself, and Jalalpur village, situated on the immediate opposite bank of the river, and a few villages down stream within two miles of Mondair, escaped entirely. The bulk of the epidemic spread was, therefore, by human contact, or through flies, which were particularly prevalent at the time.

#### RESULTS OF TREATMENT WITH BACTERIOPHAGE.

At the time of the outbreak there was a sufficient supply of bacteriophage available with the village stockists for the treatment of earlier cases. In addition,

#### GRAPH

*Cholera epidemic, village Mondair, Habiganj.*



bacteriophage was distributed to each household on the 16th April, so that it might be available for immediate use in any household. The first case (3rd April) had

bacteriophage about six hours after the onset of symptoms. This patient died four days later.

In all there were 65 attacks with 33 deaths (*see Graph*). Other cases, as mentioned above, are not included in this analysis. Of the 65 cases, seven cases received no treatment. It appears they were prevented from taking any medicine by a Pir who had visited the village. Fifty-eight cases were, therefore, treated with bacteriophage. Of these, two cases besides having bacteriophage, had some mixtures and powders as well, given by a private practitioner of Baniachung, and one case took bacteriophage in hot water. Omitting these three cases the results obtained in the 55 cases treated with bacteriophage only were analysed :—

TABLE I.

	Total cases.	Deaths.	Percentage mortality.
Treatment with bacteriophage.	55	27	49
No treatment ..	7	3	..

Some cases had bacteriophage immediately and others a few hours after the onset of symptoms. That there was no marked difference in the mortality rates in these sub-groups is shown below :—

TABLE II.

Administration of bacteriophage.	Cases.	Deaths.	Percentage mortality.
Immediately ..	39	18	46
Within 6 hours ..	14	7	50
12 to 18 hours ..	2	2	100

The time factor being of importance in bacteriophage as well as any other form of treatment, when dealing with a virulent infection it is necessary to ascertain whether bacteriophage had sufficient time to act and be of some therapeutic value.

To show this factor the 27 deaths in the 'phage group are further analysed in Table III :—

TABLE III.

Day of death after bacteriophage administration.	Number of deaths.	Remarks.
1st day ..	8	5 had 'phage immediately.
2nd day ..	6	4 had 'phage immediately.
3rd day ..	5	3 had 'phage immediately.
4th day ..	4	2 had 'phage immediately.
5th day ..	1	} had 'phage immediately.
6th day ..	1	
7th day ..	1	
9th day ..	1	

From the above table it appears that of 27 cases that died, 19 had lived for 24 hours or more after the administration of bacteriophage. In spite of early administration of bacteriophage the mortality rate in this epidemic was high.

The question whether bacteriophage had reduced the infectivity of the vibrio was then investigated. In all there were 35 primary cases, of which 15 gave rise to 22 secondary cases. Full details of these 15 primary cases and their secondaries are given in Table IV :—

TABLE IV.

Primary case.	Recovered or died.	Bacteriophage when administered.	Number of secondary cases.	Number of days after the primary case.	Population in the house.
1	Died 1st day ..	Immediately	1	3 days	5
1	Died 4th day	6 hours after onset	2	5 days and 7 days	6
1	Died 7th day	Immediately	2	3 days and 4 days	10
1	Recovered ..	Immediately	1	2 days	6
1	Recovered ..	Immediately	2	2 days and 3 days	8
1	Died 3rd day	Within 5 hours	1	4 days	7
1	Recovered ..	Immediately	3	3 days 3 days 7 days	8
1	Recovered ..	6 hours	1	7 days	10
1	Recovered ..	Immediately	1	5 days	5
1	Recovered ..	Immediately	1	8 days	8
1	Recovered ..	Immediately	1	3 days	6
1	Recovered ..	5 hours	1	5 days	5
1	Recovered ..	5 hours	2	8 days and 10 days	7
1	Died 2nd day	Immediately	1	3 days	6
1	Died 6th day	Immediately	2	1st day, 5 days	7

It will be seen that nine cases out of these 15 primary cases had recovered. Twenty-one secondary cases developed from two to eight days and one in 10 days after the primary case. All the primary cases had received bacteriophage within five hours from the onset of symptoms. It is interesting to note that of seven cases which did not receive any treatment, three were primary cases which did not give rise to any secondaries. The figures in the no treatment group are, however, too small for any significant comparison to be made.

It appears that bacteriophage had had little influence in this epidemic in reducing the infectivity of the vibrio to actual contacts.

#### COMMENTS.

This is the second village epidemic in which the bacteriophage had apparently failed to reduce the case mortality appreciably. The first outbreak was at Radhala in the Nowgong District in 1934 with 22 attacks and 16 deaths.

As is well known, the case mortality varies in different epidemics, and, in the absence of a significant number of cases receiving no treatment with which to judge the virulence of the epidemic, it would be difficult to determine whether bacteriophage had really failed or not. We have in this connection three instances of epidemics of cholera in this province, viz., the Sibsagar, Darrang and Cachar epidemics which on investigation showed varying degrees of virulence (Morison *et al.*, 1933, 1934). The mortality rates in these epidemics are shown in Table V:—

TABLE V.

Epidemics.	CASES RECEIVING NO TREATMENT.		BACTERIOPHAGE TREATMENT WITHIN 48 HOURS OF ONSET.	
	Cases.	Percentage mortality.	Cases.	Percentage mortality.
Sibsagar ..	399	48.1	124	23.4
Darrang ..	138	83.3	67	44.5
Cachar ..	958	70.0	641	21.2

If then bacteriophage had been used for the treatment of *all cases* in the Darrang epidemic (as was done in the Mondair outbreak) and the total case mortality had been reduced to 44 per cent as in the bacteriophage cases, and these figures were then compared with the 48.1 per cent mortality rate in the untreated group of the Sibsagar epidemic, the beneficial results of the bacteriophage treatment in Darrang would not have been apparent. In the Darrang outbreak we must have been dealing with a vibrio of different virulence than that of the Sibsagar epidemic. The mortality rate in the Mondair outbreak would suggest that the virulence of the responsible vibrio was comparable to that of the Darrang epidemic. Indeed the Mondair outbreak and the Darrang epidemic have one feature in common which

throws light on the equally virulent nature of the two epidemics, viz., the 'mortality by periods'.

In the Darrang epidemic, which lasted for approximately seven weeks, there was no general diminution in mortality rates on successive periods. It was 87 per cent in the beginning, and 75 per cent towards the end of the epidemic. In the Mondair outbreak which lasted for three weeks, the case mortality was equally high at all periods of the epidemic, but the epidemic was of shorter duration and was localized.

In the Sibsagar epidemic, however, a gradual diminution in case mortality occurred among the untreated group varying from 60 per cent to 20 per cent during successive stages, whereas in the bacteriophage treated group it was the same—20 per cent throughout the epidemic. If the bacteriophage treatment then tends to make the mortality curve flat, to that extent it appears that bacteriophage may have acted in Mondair, as it did in Sibsagar and Darrang epidemics, but no comparisons can be made with an untreated group in Mondair because of the paucity of cases in this classification. It was unfortunate that no bacteriological investigations could be made during the epidemic, as the report of this epidemic was received late, but strains of vibrios collected from convalescent cases have proved atypical non-agglutinating strains.

From the 65 reported cases of cholera analysed in the body of the paper it might be judged, in spite of the question of the virulence of the infection which has been discussed, that bacteriophage treatment has given no apparent striking results. However, as has been stated previously, there had occurred during the period of this epidemic, a large number of cases of diarrhoea which had received bacteriophage. These were not reported as cholera. To ascertain whether these were actually cases of cholera, specimens of blood of six such diarrhoea cases were obtained for agglutination tests. Of these six diarrhoea cases, five gave a positive agglutination against a known strain of cholera vibrio (653) in some cases up to the titre of 1 to 100. As a control, sera of two known cholera cases were examined and proved positive in 1 to 100 in one case, and 1 to 300 in another. Cholera vaccine has not been used in this locality since 1932. It must be concluded, therefore, that these diarrhoea cases were really cases of cholera.

We realize, however, that such unreported mild cases must be present in all epidemics. The significance of this factor in evaluation of the bacteriophage treatment is discussed in another paper (Pandit *et al.*, 1936).

<sup>1</sup> In view of these findings it would be premature to say, in the present state of our knowledge, that bacteriophage had absolutely no effect in altering the character of the epidemic in some ways.

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## IMMUNIZATION AGAINST PLAGUE WITH LIVE VACCINE.

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A FORMER paper dealt with the experimental results obtained with various dead vaccines in guinea-pigs, wild house rats (*R. r. diardi*), white laboratory rats, mice and monkeys (*Macac. cynomolgus*). It appeared that the nature of the experimental animal was by far more essential to the results than the nature of the vaccine used. No dead vaccine whatever conferred satisfactory protection to guinea-pigs and wild rats—the test animals most susceptible to plague infection.

I, therefore, turned to a live vaccine, just as also did Strong (1906, 1907) who, after equally disappointing results with dead vaccine, more successfully carried on his investigation with a live strain. While Strong (*loc. cit.*) used the artificially attenuated strain 'Maassen', already experimented with by Kolle and Otto (1903 *a* and *b*, 1904) some years previously, I selected a strain cultivated from a plague rat (found dead at Tjiwidej, chief town of a district near Bandoeng) which after rat-passage and storage in deep serum-agar stab-culture 4 months later casually was found to have entirely lost its virulence: subcutaneous injection of half an agar-slope culture into rats and guinea-pigs provoked no symptoms whatever. At first I intended to use another available strain, cultivated in 1920 from the bubo of a man suffering from plague in Mid-Java, and which had also been stored in deep serum-agar stab-culture since 1924. In January 1930, 10 years later, this strain 'Java' showed markedly reduced virulence; the wild rat withstood half an agar-culture, a dose, however, still lethal for guinea-pigs. One year later an entirely avirulent variant could be sub-cultured. Since, however, in some preliminary experiments its immunizing potency seemed below that of the above-mentioned strain 'Tjiwidej' I decided for this so quickly, spontaneously attenuated strain.

All experiments were carried out in exactly the same way as already described for those with dead vaccine: one subcutaneous injection (or three injections at a week's interval) of a dose expressed in fractions of an agar-slope culture after 48 hours' growth at 30°C., and subsequent subcutaneous infection 3 weeks after the (last) immunizing dose with a body strain as applied by Stevenson and Kapadia (1911), viz., 1 c.c. of a 1/100,000th dilution of 250 mg. spleen of a freshly dead



plague rat, with the difference that the dilution administered was usually 4 times higher and suspended in 0.1 c.c. instead of in 1 c.c. of the vehicle. As a rule the highly virulent strain 'Preanger' was used which is constantly stored as mentioned above. For each experiment after guinea-pig sub-passage some rats were infected and, as determined by microscopic examination, the most heavily infected spleen was selected. The number of viable and highly virulent plague bacilli of the test dose for rats averaged 50,000 and for the guinea-pigs a ten-fold dose was applied; the observation period allowed was 30 days\*.

Though the first experiment (partly with 3 injections—1/50th, 1/10th and 1/5th agar-culture—and partly with one injection of 1/5th culture) gave rather satisfactory results in rats (15 : 14/1) as well as in guinea-pigs (15 : 15/0), in further experiments only 57.7 per cent of the rats (45 : 26/19) and 25 per cent of the guinea-pigs (20 : 5/15) survived, irrespective of the dosage administered and the number of injections given. As the strain had been continuously sub-cultured on agar since the first experiment, the possibility of bacterial dissociation as a cause of this lack of success was suggested: such appeared indeed to be the case. In agar-plate cultures two differently shaped colonies could be easily distinguished. According to the usual terminology these variants were designated as 'smooth' and 'rough', a distinction to be discussed later in connection with the dissociation phenomenon in *Bacillus pestis*. With these two variants which, stored in stab-culture, maintained their characteristics, extensive series of tests were carried out in rats and guinea-pigs with gradually higher dilutions of one dose only (cf. Table I).

The results obtained with these variants were rather divergent: while with the 'Tjiwidej' smooth variant in a dose of 1/5th to 1/1,000th agar-culture 75 to 100 (average 85) per cent of the rats survived, a survival rate of only 24 to 50 (average 32.5) per cent was attained with the 'Tjiwidej' rough variant. When using higher dilutions for immunization, the effect was less favourable but an equally striking difference was noted, viz., 70.8 per cent survival against 22 per cent with a vaccinating dose of 1/5,000th to 1/100,000th culture. Even with an immunizing dose of 1/1,000 millionth culture of 'Tjiwidej' smooth (containing about 5 viable bacilli) 2 out of 10 rats survived†.

In guinea-pigs the difference of antigenic potency was even more striking, though in this test animal a higher vaccinating dose was required to give optimum differential results: 80 to 100 (average 94) per cent survival with 1/5th to 1/1,000th culture of 'Tjiwidej' smooth against 15 to 30 (average 23) per cent in the corresponding rough series.

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\* Since sufficiently good results had already been produced in white laboratory rats and monkeys with a dead vaccine, even better results could be expected with a live vaccine in these animals. As such was indeed the case (white rats 15 : 15/0, monkeys 35 : 33/2 with a ten- and hundred-fold infecting dose respectively) I confined myself in further experiments to guinea-pigs and wild rats.

† This is the more striking since these results are superior to those with the highly virulent strain 'Preanger': of some 42 rats, which had survived infection with decreasing doses of this strain in experiments described in my former paper, after about 2 months only 2 resisted re-infection with the usual test dose of the same strain. These two rats had survived a first infection with the same test dose containing round about 30,000 viable bacilli; the remaining 40 rats all died acutely, though the first (vaccinating) infection for most of them had been more than 5 viable organisms; 10 had been inoculated with 5, 7 with 20, 6 with 30, 5 with 50, 7 with 200, 3 with 500 and 2 with 2,000 viable bacilli. This experience endorses the contention often expressed, but lacking definite evidence, that the immunizing potency of a strain does not in the first place depend on its virulence.

TABLE I.

Vaccination dose (agar-culture).	HOUSE RAT.				GUINEA-PIG.			
	Results Tjiw. smooth.	Percentage survival.	Results Tjiw. rough.	Percentage survival.	Results Tjiw. smooth.	Percentage survival.	Results Tjiw. rough.	Percentage survival.
1/5	(20) 20: 15/5	75	(20) 20: 7/13	35	(21) 21: 20/1	95	(20) 20: 3/17	15
1/25	(10) 10: 10/0	100	(10) 10: 5/5	50	(9) 9: 9/0	100	(11) 11: 3/8	27
1/50	(20) 20: 19/1	95	(20) 20: 8/12	40	(20) 20: 20/0	100	(20) 20: 5/13	25
1/100	(25) 25: 20/5	80	(25) 25: 6/19	24	(35) 35: 35/0	100	(35) 35: 11/24	31
1/500	(10) 10: 10/0	100	(10) 10: 4/6	40	(25) 24: 20/4	83	(25) 24: 4/20	16.5
1/1,000	(50) 50: 41/9	82	(50) 50: 14/36	28	(10) 10: 8/2	80	(10) 10: 2/8	20
1/5,000	(10) 10: 7/3	70	(10) 10: 1/9	10	..	..	..	..
1/10,000	(20) 19: 15/4	79	(20) 20: 5/15	25	(20) 19: 10/9	52.5	(20) 20: 0/20	0
1/100,000	(20) 19: 12/7	63	(20) 20: 5/15	25	(10) 10: 2/8	20	(10) 10: 0/10	0
1/1 ion	(20) 19: 13/6	68	(20) 20: 1/19	5	..	..	..	..
1/10 ion	(10) 10: 5/5	50	(10) 10: 0/10	0	..	..	..	..
1/100 ion	(10) 9: 1/8	11	(10) 10: 0/10	0	..	..	..	..
1/1,000 ion	(10) 10: 2/8	20	(10) 10: 0/10	0	..	..	..	..
1/5-1/1,000	(135) 135: 115/20	85.2	(135) 135: 44/91	32.5	(120) 119: 112/7	94.0	(121) 120: 28/92	23
1/5,000-1/100,000	(50) 48: 34/14	70.8	(50) 50: 11/39	22.0	(30) 29: 12/17	41.4	(30) 30: 0/30	0
1/1 ion-1/1,000 ion	(50) 48: 21/27	43.7	(50) 50: 1/49	2.0	..	..	..	..
Controls	..	90: 0/90 = 0 per cent survival.	..	80: 0/80 = 0 per cent survival.	..	..	..	..

Note.—(20) 19: 15/4 = 20 vaccinated, 19 infected (one dead before infection), of which 15 survived and 4 died.

The superiority of the smooth antigen was shown not only by the lower mortality rate but also by the longer period between infection and death. This averaged 8.5 days in the 45 rats dead in the smooth series (immunized with 1/5th to 1/10 millionth culture) against 6 days and 4.1 days in the 159 and 90 rats dead in the rough series and among the controls respectively. This difference is also present in guinea-pigs though less pronounced, as the period of infection in controls, averaging 6 days, exceeds that in rats; for the rough series it averaged 8, for the smooth series 9.4 days. In the following table the results of all the experiments that were carried out with 'Tjiwidej' smooth in doses down to 1/1,000th cultures during the years 1930-1935 are collected. Guinea-pigs inoculated with a full-agar culture served as a control of the innocuousness of the vaccine prepared with this strain for inoculation in man. Some 76 guinea-pigs injected for the same purpose with a full broth culture (5 c.c. corresponding to round about 1/5th agar-culture) cultivated from the original stab-culture are not included; of these animals 96 per cent survived (76 : 73/3).

TABLE II.

Vaccination dose.	HOUSE RAT.		GUINEA-FIG.	
	Results.	Percentage survival.	Results.	Percentage survival.
2 .. ..	(10) 10 : 9/1	90	.. ..	..
1 .. ..	(10) 10 : 8/2	80	(97) 97 : 93/4	95.8
1/5 .. ..	(20) 20 : 15/5	75	(21) 21 : 20/1	95
1/25 .. ..	(10) 10 : 10/0	100	(9) 9 : 9/0	100
1/50 .. ..	(60) 60 : 52/8	86.6	(30) 30 : 29/1	96.6
1/100 .. ..	(340) 340 : 272/68	80	(265) 264 : 234/30	88.6
1/500 .. ..	(10) 10 : 10/0	100	(25) 24 : 20/4	83.3
1/1,000 .. ..	(50) 50 : 41/9	82	(10) 10 : 8/2	80
TOTALS ..	(510) 510 : 417/93	81.7	(457) 455 : 413/42	90.7
Controls ..	200 : 2/198	1.0	170 : 0/170	0.0

In rats a survival rate of more than 80 per cent and in guinea-pigs of more than 90 per cent was attained without any loss through inoculation; only a few animals died during the vaccination period from some intercurrent disease. Rats can resist even 1 to 2 agar-cultures without any ill effect, whereas the injection of the same dose of the same suspension killed by heat at 60°C. kills 35 per cent within 48 hours from toxæmia (20 : 13/7). This paradoxical phenomenon may be explained by the fact that a fresh suspension of live bacilli of this strain is but slightly or not at all toxic so that the organism can resist the endotoxins gradually produced by the slowly dying bacilli. When, on the contrary, a suspension is quickly killed by heating at 60°C., all endotoxins are produced simultaneously and the organism is unable to meet so highly toxic a dose. Besides, the possibility is suggested that the heating of endotoxins may give rise to products of an even more toxic nature. Death from toxæmia can also be provoked by a fresh live suspension provided it is

injected intraperitoneally, since by this mode of injection the bacilli are, at least partly, subject to quick destruction and the rat dies from toxæmia through the mass-production of endotoxins accordingly. That the strain 'Tjiwidej' is absolutely avirulent is clear, not only from the total absence of deaths in the experiments mentioned above but also from the following test made in a series of 4 rats subcutaneously inoculated with a full agar-culture, of which 2 were killed after 24 and 48 hours respectively and two served as controls. The strain, sub-cultured from the organs of the first two rats, was further sub-passaged in 4 rats with the same dose; even after the tenth sub-passage the controls survived in perfect health.

Some experiments were carried out to investigate the question as to whether seeding at 37°C. could further raise the antigenic potency of the strain. According to Schütze (1932) the *Bacillus pestis* possesses two antigens, a somatic antigen and one contained in the gelatinous envelope, the latter being of paramount importance for the efficiency of a vaccine. Its development should be highly favoured by growth at 37°C. The table below shows that seedings at 30°C. or 37°C. yield practically the same results; two different samples of the vaccine were used, the first one immediately after preparation and also 7 days afterwards, the other immediately.

TABLE III.

Vaccination dose.	HOUSE RAT.			GUINEA-PIG.		
	30°C.	37°C.	Controls.	30°C.	37°C.	Controls.
	Results.			Results.		
1/50 after preparation ..	10 : 7/3	10 : 9/1	} 10 : 0/10 {	9 : 8/1	10 : 8,2	} 10 : 0/10 {
1/50 7 days later ..	10 : 9/1	10 : 9/1		10 : 8/2	10 : 10,0	
1/100 after preparation ..	10 : 8/2	10 : 9/1	10 : 0/10	10 : 10/0	10 : 7/3	10 : 0/10
TOTALS .. ..	30 : 24/6	30 : 27/3	20 : 0/20	29 : 26/3	30 : 25/5	20 : 0/20
Percentage survival ..	80	90	0	90	83	0

With a view to the evaluation of the results obtained with the strain 'Tjiwidej' those of other investigators with live vaccine will be briefly summarized here. Already in 1897 Albrecht and Gohn (1900), members of the Austrian Plague Committee, who went to Bombay shortly after the outbreak of plague, immunized some guinea-pigs and rats (presumably *R. norvegicus*) with spontaneously attenuated strains and tested them after a vaccination period of some weeks. The results were fairly good but, since the strains used still caused deaths from plague and the number of experimental animals was small and, last but not least, the number of controls was absolutely insufficient, these tests are of slight value. This criticism holds good for those of Yersin and Carré (1900) also: the strain they used gave rather good results in rats (white rats?) but it also caused deaths from plague. This disadvantage could be met by using older cultures of 40 to 50 days' growth,

at the expense however of the results attained ; also the number of controls used in their few experiments was too small.

Of more value are the investigations of Kolle and Otto (*loc. cit.*) made in extensive series of experiments with different strains, partly spontaneously and partly artificially attenuated. The spontaneously attenuated strains, however, often caused buboes and occasionally deaths from sub-chronic plague. The artificially attenuated strains—all derived from the so-called strain 'Maassen' [after Maassen (quoted by Kolle and Otto, *loc. cit.*) who attenuated this strain in a way still unknown] also caused deaths from plague in guinea-pigs. Only the strain 'Maassen V', grown at a temperature of 40°C. to 41°C. over a long period, was absolutely avirulent for guinea-pigs even when injected subcutaneously in a dose of 1 to 2 agar-cultures, a dose able to kill them from toxæmia only when injected intraperitoneally. Whether the cause of death in rats must be accounted for by plague or by toxæmia, is not clear from the text. Results were more favourable in guinea-pigs than in rats, the survival rate for the former averaging 72, for the latter only 35 per cent.

The most accomplished research work was carried out by Strong (1906, 1907) some years later with the same strains 'Maassen alt' and 'Maassen V':. Numerous experiments were made in guinea-pigs and monkeys (*Cynomolgus philippinensis*) without any deaths from plague notwithstanding a vaccinating dose of 1 to 2 agar-cultures. This suggests the possibility that these strains underwent further spontaneous attenuation in the course of time ; the few animals lost during the vaccination period apparently died from toxæmia. The results obtained by Strong (*loc. cit.*) surpassed by far those of Kolle and Otto (*loc. cit.*). It must, however, be noted that his experiments are not exactly comparable with those of the latter investigators who usually chose a smaller vaccinating dose and allowed a longer vaccination period in many of their experiments. The fact remains that Strong, considering the marked difference between the effect with live and dead vaccine, rightly concluded that vaccination with live bacilli deserves preference. After having stated the innocuousness of the strain 'Maassen' for man by inoculation of some 200 persons with this vaccine, that even in a dose of a whole agar-culture did not give rise to any severe reactions, Strong expressed himself as a convinced supporter of vaccination with live vaccine. It was not until a quarter of a century later, however, that its application was realized.

In 1927 Pirie carried out some experiments in Gerbilli (*G. lobengulæ*) with a view to investigating whether immunization with live avirulent plague bacilli was more efficient than with a dead vaccine, which had given rather unsatisfactory results. Of the 4 strains he used one appeared to have maintained its full virulence : all test animals vaccinated with it died acutely from plague. Though they did not cause deaths from plague the 3 other strains nevertheless gave a high mortality rate during the vaccination period, presumably from toxæmia. When tested by infection after 9 days, one strain appeared an absolute failure and the other two gave survival rates of 33.3 and 50 per cent respectively, a result, though superior to that with dead vaccine, not altogether satisfactory in view of a percentage mortality from toxæmia of 20 to 25.

Finally, almost at the same time, two communications were published on this subject. one by Girard and Robic (1934) (read by Mesnil before the

TABLE IV.

Author.	Strain.	Test animal.	Dose (agar-culture).	VACCINATION.		INFECTION.		CONTROLS.	
				Results.	Percentage loss.	Results.	Percentage survival.	Results.	Percentage survival.
Kolle and Otto <sup>1</sup> ..	'Maassen alt'	Guinea-pig	1/100-1/20	32: 23/9	28	22: 17/5	77.3	37: 0/37	0
	" V	"	1/10-3	26: 26/0	0	21: 14/7	66.6		
	" alt	White rat	1/100-1/10	20: 20/0	0	19: 8/11	42.1	31: 1/30	3.2
	" V	"	1/10-1/2	20: 15/5	25	15: 4/11	26.6		
Strong ..	'Maassen alt'	Guinea-pig	1	30: 28/2	6.6	28: 25/3	89.3	70: 0/70	0
	" V	"	1	51: 51/0	0	51: 35/16	68.8		
	" alt	Monkey	1/2	49: 43/6	12	43: 30/13	70	80: 7/73	8.7
	" V	"	1/2	43: 42/1	2.3	42: 21/21	50		
Otten ..	'Tjiwidej' sm.	Guinea-pig	1/1,000-1/100	300: 298/2	0.6	298: 262/36	87.9	180: 0/180	0
	"	"	1/50-1	233: 233/0	0	233: 224/9	96.1		
	"	House rat	1/1 ion-1/100	460: 457/3	0.6	457: 363/94	79.4	200: 2/198	1
	"	"	1/50-2	110: 110/0	0	110: 94/16	85.4		

'Académie de Médecine' in Paris in the session of 26th June, 1934), the other by me (Conference of the Netherlands' Association for Tropical Medicine in Amsterdam on 25th March, 1934). Girard and Robic used a strain, designated 'E. V.' and isolated in 1926 from a human case of bubonic plague in Madagascar, since grown at room temperature (18°C. to 25°C.) and sub-cultured monthly on agar. Six years later, in 1932, this strain appeared to be absolutely avirulent for guinea-pigs when injected subcutaneously in a dose of a whole agar-culture; white mice and rats, however, could resist only a relatively small dose (not exactly indicated by the authors); with increased doses they died from toxæmia just as did 20 to 30 per cent of guinea-pigs when inoculated intraperitoneally with  $\frac{1}{3}$ rd agar-culture or more. However, their experience that guinea-pigs, when vaccinated with this strain, showed to be absolutely immune against cutaneous infection with virulent plague matter (spleen, lung, etc.), was of paramount importance.

To facilitate a general view on the subject the results of Kolle and Otto—as far as immunization by subcutaneous injection with a live avirulent vaccine is concerned—of Strong and mine are collected in the synoptic table above. The experiments of Pirie are only few in number, while those of Girard and Robic cannot be quoted here because of particulars not being mentioned and data about number of test animals being lacking.

The results obtained with the strain 'Tjiwidej' undeniably compare favourably with those with the strain 'Maassen', especially when the far smaller dose applied in the majority of the test animals and the fact that immunity is less easily produced in wild rats than in white rats and monkeys, are considered.

As to particulars about the immunity produced by the smooth strain 'Tjiwidej' I investigated into the period required for its development to a maximum and into the period this was maintained. In Table V the results of all experiments in rats and guinea-pigs, infected in the usual way 1 to 21 days after immunization with a dose of 1/100th agar-culture, are collected; the figures represent the total of 5 series in rats and of 3 in guinea-pigs:—

TABLE V.

Infection after number of days.	DEVELOPMENT OF IMMUNITY IN			
	HOUSE RAT.		GUINEA-FIG.	
	Results.	Percentage survival.	Results.	Percentage survival.
0	50 : 0/50	0·0	30 : 0/30	0·0
1	40 : 13/27	32·5	30 : 1/29	3·3
2	40 : 9/31	22·5	29 : 4/25	13·7
3	45 : 16/29	35·5	30 : 3/27	10·0
5	45 : 25/20	55·5	29 : 13/16	44·8
7	45 : 23/22	51·5	30 : 14/16	46·6
10	45 : 30/15	66·6	29 : 24/5	82·7
14	50 : 42/8	84·7	26 : 23/3	88·4
21	49 : 43/6	87·7	30 : 27/3	90·0

It appears that an appreciable degree of immunity has already developed within a few days, especially in rats, which, however, becomes significant only after some 5 to 7 days and gradually increasing does not reach its maximum until 2 to 3 weeks. This does not tally with the experience of Stevenson and Kapadia (1911), who noted that immunity developed quickly in rats after injection of  $\frac{1}{4}$  c.c. of the Haffkine vaccine, reached its maximum after 2 to 3 days to drop, though slowly, after that period. It must be borne in mind, however, that the Haffkine vaccine confers on the rat a relatively low degree of immunity, widely differing in consecutive series of experiments (in their first experiment 28.2 per cent survived after 3 days, in their second 62.5 per cent) and simultaneous tests may give divergencies amounting to even 100 per cent. It is obvious, therefore, that definite conclusions as to the development of immunity can hardly be drawn from their results. In my opinion, a gradual increase of immunity as shown in my experiments with the strain 'Tjiwidej', that agrees with the experience of Yersin and Carré (*loc. cit.*) just as with that of Girard and Robic (*loc. cit.*) and with the gradual appearance of anti-bodies demonstrable *in vitro*, such as agglutinins, is more in harmony with the facts.

In this connection it is a matter of doubt as to whether the immunity noted in the first days after vaccination be of an exclusively specific nature; especially the fact established by Stevenson and Kapadia that infection some few hours after vaccination gave a higher survival rate against that of controls, suggests that non-specific factors play a part, viz., an aspecific increase of resistance as presumed by Pfeiffer and Issaëf (1894). Anyhow it is clear from the experiments of Stevenson and Kapadia and from mine that a so-called 'negative phase' is not shown in accordance with the experience gained in man. From the data collected by Bannerman (1901-1902) it appears that mortality in those inoculated with the Haffkine vaccine is always lower than in those not inoculated, even in the last days of the incubation period and even when the first symptoms of the disease appear on the day of vaccination or are already manifest at that moment. In vaccination of man with live vaccine a negative phase could not be observed either, as the data concerning this subject will show.

As to the duration of immunity, this has been ascertained in rats and guinea-pigs. The wild rat, however, proved an unsuitable test animal in this respect, since, when imprisoned in small cages of 25 cm. by 12 cm. by 12 cm., it begins to languish after some months so that an increasingly higher loss of rats occurs after some 4 to 5 months. The experiments with rats, therefore, were discontinued after 6 months; though guinea-pigs were more suitable, the test with them could not be extended over more than 9 months. In Table VI the results are collected: the vaccinating dose was  $1/50$ th agar-culture, partly inoculated by one and partly in 3 injections at a week's interval; 3 weeks after the last injection the first series was infected with the usual test dose (ten-fold dose for guinea-pigs), 3 months later the second series and so on.

After 6 months the immunity of rats had decreased considerably; their bad health, however, must be taken into consideration. In guinea-pigs, too, immunity had appreciably dropped: after 6 months it was still fairly satisfactory, after 9 months, however, it had markedly decreased. That it has not yet entirely faded away appears from the lower mortality rate as compared with controls (75 per cent against 100 per cent) and from the longer period between infection and death (8.2



against 6.1 days). As a matter of course these experiments have little or no practical significance for the solution of the question of how long immunity may persist in man; this can be solved only by the course of an epidemic after vaccination.

TABLE VI.

Infection after	DURATION OF IMMUNITY IN					
	HOUSE RAT.			GUINEA-PIG.		
	VACCINATED.		Controls.	VACCINATED.		Controls.
	1 injection.	3 injections.		1 injection.	3 injections.	
	Results.			Results.		
3 weeks ..	10 : 8/2 <sub>13</sub>	10 : 10/0		10 : 2/8 <sub>3.6</sub>	10 : 9/1 <sub>8</sub>	
3 months ..	10 : 7/3 <sub>5.7</sub>	10 : 6/4 <sub>8</sub>	10 : 0/10 <sub>3.8</sub>	10 : 8/2 <sub>17.5</sub>	10 : 10/0	10 : 2/8 <sub>10</sub>
6     "     ..	10 : 2/8 <sub>3.6</sub>	10 : 3/7 <sub>3.6</sub>	10 : 1/9 <sub>3</sub>	10 : 6/4 <sub>9.5</sub>	10 : 8/2 <sub>11.5</sub>	10 : 0/10 <sub>6.3</sub>
9     "     ..	..	..	..	10 : 2/8 <sub>8.3</sub>	10 : 3/7 <sub>8.1</sub>	10 : 0/10 <sub>6.1</sub>

The subscript figures indicate the average number of days of survival after infection.

#### VACCINATION IN MAN.

Before this vaccination could be started, it obviously was indispensable to prove the suitability of the strain 'Tjiwidej', not so much as to the possibility that it occasionally causes death in man—such being rather improbable considering the fact that guinea-pigs and rats resist inoculation of a whole agar-culture without any untoward effect—as to ascertain the nature of the reaction in response to the injection. Animal test had shown that as a rule in guinea-pigs, but rarely in rats, a well-marked infiltration develops at the site of injection. Generally this infiltrate is resorbed in a few weeks, sometimes, however, it gives rise to a sterile abscess that finally breaks without any disadvantage to the guinea-pig. If such infiltrates, or abscesses should regularly develop in man too, this would mean a conclusive objection to the application of this strain for the vaccination of the population. Fortunately the vaccine appeared quite harmless to man, as the reaction was slight beyond expectation: after inoculation of 1/50th culture (about 100 millions viable and 200 millions dead organisms) the reaction was confined to a small infiltrate with slight, if any, rise of temperature, even when the dose was increased to 1/25th culture. After some more volunteers had submitted to vaccination and had not

shown any serious local or general symptoms even with an increased dose up to  $\frac{1}{4}$ th culture, the start of a preliminary immunization of the population according to the alternating system was indicated in order to decide whether this vaccine was able to protect man against plague infection.

During the epidemic in Mid-Java, in 1921-1922, with the Haffkine vaccine prepared partly in the Haffkine Laboratory at Bombay and partly in the Pasteur Institute at Batavia, according to this system, an extensive experiment was made. More than 35,000 persons were vaccinated during those years: in each household half of the inmates were selected as far as possible according to age and sex in order to obtain an exactly comparable control group. This procedure resulted in a reduction of plague mortality to 50 per cent, undoubtedly an appreciable reduction but not sufficient to make an impress on the minds of the native population, the less so since in 1922—the year of the greatest number of vaccinations (80 per cent)—in some sub-districts, where the epidemic showed an acute rise, mortality was reduced by only  $\frac{1}{3}$ rd. These results obviously compare unfavourably with those obtained in British India, where according to many communications on the activity of the Haffkine vaccine a six- to ten-fold reduction of the mortality rate and even more is recorded. Most of these reports, however, are of little or no statistical value whatever, as was already emphasized in a former paper.

The usual mistake met with is that vaccination is confined to those willing to submit to it and that the incidence of plague in this group is compared with that amongst those opposed to immunization. As a matter of course the last group consists mostly of people of little or no education at all or contains adherents of some special creed, living accordingly under particular conditions. In British India especially with its strict caste system, the standard of living and the housing conditions will widely differ. From this it will be clear that, as a rule, the two groups are far from comparable. A further statistical error arises from the circumstance that vaccination may proceed slowly and is not completed until the end of the epidemic. During its increase—in the period of highest mortality—the victims mainly prevail among unvaccinated; consequently results will be the more flattered the later vaccination is started and the more slowly it proceeds. Even when vaccination is started opportunely, statistics may be flattered if plague does not spread diffusely but is more or less localized and those vaccinated may, for the greater part, dwell in the slightly or not infected quarters. In short, in almost none of the available plague statistics has the erratic spread of plague been taken into consideration and, as a rule, wholly heterogeneous groups have been compared. To ensure reliable statistics an exactly comparable control group is required and this is ensured only by the alternating system of vaccination in order to prevent volunteers from being opposed to those who refuse, as well as a small group of vaccinated from being compared with a large one of unvaccinated available from different phases of the epidemic and living in different quarters. This is feasible only by an equal partition of the population according to sex and age up to its smallest social unit, the household. Only in this way are two similar groups ensured, living under the same conditions and exposed to the same risk of infection during the same period. Though the preliminary measures as well as the execution of such an alternating vaccination scheme require a great deal of time and effort, it must be emphasized that only this method guarantees two exactly comparable groups in order that a marked difference of the mortality rates in favour of those

vaccinated can be explained only by their artificially increased resistance owing to vaccination.

The preliminary vaccination mentioned above was carried out in two sub-districts of the Regency of Bandoeng, viz., Bandjaran and Batoedjadar. After registration of the houses and their inmates of all 25 villages (desas), 37,000 persons were alternately vaccinated in the course of one month (from 3rd November to 6th December, 1934). This vaccination in each village and its accessory hamlets, forming a unit, was accomplished in one day unless too scattered. After it was finished full attention was paid to all cases of death since, apart from the diagnostic difficulties, it is impracticable to trace all living cases of plague. In all deaths recorded spleen and lung puncture was performed and smears were made of both, which in addition to spleen matter were sent to the Pasteur Institute, where the diagnosis was ascertained by microscopic examination and, in doubtful cases, by animal test. Over a period of 5 months (21 weeks) more than 1,200 cases of death were checked in this way and 356 cases of plague detected; the partition of these cases among vaccinated and unvaccinated is shown in Table VII:—

TABLE VII.

Sub-district.	BUBONIC PLAGUE.					
	VACCINATED.			UNVACCINATED.		
	Number.	Deaths.	Mortality per mille.	Number.	Deaths.	Mortality per mille.
Bandjaran ..	18,479	28	1.5	20,669	103	4.9
				*2,755	10	3.4
				17,914	93	5.1
Batoedjadar ..	18,956	10	0.5	24,088	110	4.6
				*2,519	3	1.2
				21,569	107	5
Totals ..	37,435	38	1.01	44,757	213	4.75
				*5,274	13	2.46
				39,483	200	5.05

\*Absentees from causes other than disease, childbirth or old age.

The cases occurring in the first week after vaccination (21, of which 4 were among the vaccinated) are eliminated since, on the one hand, these cases concern vaccinated people in whom immunity had not yet fully developed, so that their death from plague cannot be considered as any reflection on vaccination; when adding them to the number of failures the efficiency of the vaccine would be unjustly minimized. On the other hand, deaths from plague among those not vaccinated is partly due to persons falling ill just before vaccination; if these were added to the statistics, the effect of vaccination would be flattered. Cases of primary pneumonic plague are omitted also (84 cases, of which 36 were among the vaccinated) on the presumption, based upon data available from literature and personal research, that this vaccination could not be expected to guarantee any protection against ærogenic infection, at least when subcutaneously administered at the relatively small dose adhered to till now\*.

The total number of deaths from bubonic plague ascertained in the 21 weeks period mentioned above amounted to 251, of which 38 occurred in vaccinated and 213 in the control group, giving a mortality rate of 1·01 against 4·75 per mille or a reduction of mortality in vaccinated to almost 20 per cent. On closer examination of the particulars of both the sub-districts, however, it appears that they are widely divergent. In Bandjaran mortality is reduced to only 30 per cent (1·5 : 4·9) against almost 10 per cent in Batoedjadar (0·5 : 4·9). This striking contrast must be explained by unequal prevalence of pneumonic plague in these two sub-districts. In Batoedjadar pneumonic plague was confined to some local foci, the number of cases amounting to only 16; in Bandjaran, however, shortly after vaccination was finished, a violent outbreak was recorded in one village giving rise to dissemination in the neighbourhood that could be checked only after some months. The total number of cases amounted to 68, i.e., more than 4 times that of Batoedjadar. These figures relate exclusively to those cases which could be clinically or epidemiologically ascertained; as a matter of course, in addition to these, many cases will have occurred that could not be differentiated. As a rule the informants are not able to discriminate incidental cases of primary pneumonic plague from bubonic plague. Microscopic examination is not conclusive either, since smears typical of primary pneumonic plague in the absence of plague bacilli in the spleen are as rare as is the reverse in bubonic plague: between these two typical findings numerous transitions exist, differences being merely quantitative and so their interpretation must necessarily be arbitrary. These sporadic and undetected cases of primary pneumonic plague will range among those of bubonic plague and, since pneumonic plague will prevail equally in both groups, it is obvious that even a small number of these cases is apt unfavourably to affect statistics of bubonic plague, i.e., of a disease the incidence of which markedly decreases among successfully vaccinated as compared with non-vaccinated people.

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\* After the violent epidemic of primary pneumonic plague in Manchuria (1911-1912) Strong endeavoured to immunize a great number of monkeys against ærogenic infection with the same avirulent strain as used in his former plague experiments: over 90 per cent of them died from pneumonic plague. Guinea-pigs inoculated with the strain 'Tjiwidej', when dying after a prolonged period of illness, as a rule show symptoms of secondary pneumonic plague, the spleen on the whole having a normal aspect and repeatedly yielding a negative result on microscopic examination and also occasionally even when sub-cultured. In immunized rats that die ten days or more after infection this syndrome is usually found too, thus supporting the fact that even in immune test animals the lungs remain a *locus minoris resistentiæ*.

Apart from the unfavourable influence of overlooked cases of pneumonic plague in the sub-district Bandjaran, the effect of this trial is minimized by two further factors: in the first place by the relatively high number of absentees from causes other than disease, childbirth and old age. This group mainly concerns contract-coolies of both sexes, adults and children, who are periodically employed away at the neighbouring estates. Since, however, these estates, as a rule, are not infected, this part of the population runs no risk of infection during its absence from home, so that a lower mortality from plague may be expected among them. This indeed appears to be the case, as plague mortality amongst them was half of that among those not vaccinated, who stayed at home uninterruptedly; this means a dilution of the statistical material in favour of the unvaccinated. When these absentees to a number of more than 5,000 persons are eliminated, mortality in the control group increases to 5.05 per mille, i.e., amounts to five-fold that in vaccinated; for Batoedjadar only mortality increases to 5 per mille, exactly 10 times that of those vaccinated.

Another factor claiming attention is the different age distribution of children in both groups and its reflection on the mortality rate\*. It appeared that the average age of the vaccinated children was much higher than that of those serving as controls. In the former group the percentage of children in the age-group of 1 to 5 years amounted to only 23 against 59 in the latter. Though equal distribution according to age was devised, the greater portion of this age-group obviously escaped vaccination, probably on account of the natural inclination of the vaccinator to evade trouble and screaming. Sticker (1910) has already drawn attention to the fact that according to the data available in Bombay babies are but exceptionally infected with plague and that infection in infants under 6 years is scarcer than in older children. These data, however, are but little convincing since they concern hospital cases and it was not ascertained that the age distribution of those attended to in hospital agreed with that of those suffering from plague outside the hospital. As regards the low plague mortality of babies, this was already confirmed in the first year of the outbreak of plague in Java in 1911. Among round about 3,500 babies of both the sub-districts now concerned not a single case was noted during the period of observation. As babies were excluded from vaccination, they are eliminated from those not vaccinated.

From the statistics of these two sub-districts it further appears that mortality among unvaccinated children of 1 to 5 years was actually lower than among those 6 to 15 years old, viz.,  $34/9,693 = 3.5$  per mille, against  $39/6,735 = 5.9$  per mille (in vaccinated children the mortality rates were  $1/2,916 = 0.34$  per mille and  $9/9,762 = 0.92$  per mille respectively). The risk of infection, therefore, is markedly higher in children of 6 to 15 years. Since almost 60 per cent of the children of this age-group were vaccinated, the mortality rate of vaccinated must obviously be higher and that of unvaccinated lower than would be expected with an equal age distribution. The results of vaccination, therefore, are also unfavourably influenced by this incongruity.

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\* As to the age distribution it was noted during former epidemics in East- and Mid-Java, that mortality in both sexes was equal, at least in the country. In West-Java too this distribution appeared to be the same: a mortality of 4.81 per mille in males and of 4.71 per mille in females.

Anyhow, in my opinion, the results obtained in Batoedjadjar, where conditions were not distorted by the incidence of pneumonic plague, may be considered as normal and a reduction of mortality to 10 per cent as attainable, except for the interference of pneumonic plague. It is obvious, however, that such a reduction never will be realized, since it implies complete vaccination of the whole population which must be considered impracticable. When only 10 per cent fails, it already means a doubling of mortality or a reduction to only 20 per cent. Besides a single injection of a relatively small dose ( $\frac{1}{5}$ th agar-culture = 3 milliards plague bacilli, of which  $\frac{1}{3}$ rd is viable, for adults and half of this dose for children) guarantees protection over only a limited period roughly estimated at about 6 to 8 months. Mortality in a vaccinated area may, therefore, be expected to increase gradually till immunity be restored to its original or to an even higher level by re-vaccination. Taking into consideration all the conditions enumerated here a reduction of the mortality rate to  $\frac{1}{4}$ th may be regarded as the highest actually attainable.

In the beginning of 1933 Girard and Robic carried out a preliminary test vaccination in Madagascar with the avirulent plague strain 'E. V.' mentioned before, after having checked its innocuousness for man by subcutaneous inoculation of a number of lepers. In the first 3 months 1,600 volunteers were vaccinated and 13,000 from September to January 1934—the period of seasonal prevalence—without any untoward effect being noted. From October to December 1934, about 47,000 persons were immunized against about 60,000 serving as controls. In the course of the following 5 months 22 cases of plague were recorded in those vaccinated against 100 in the control group, viz., a mortality rate of 0.47 against 1.66 per mille, i.e., a proportion of round about 1 : 3 when eliminating cases of primary pneumonic plague, which were confined to those not vaccinated. Since this vaccination was not carried out according to the alternating method, Girard and Robic are well aware of the fact that the two groups are not exactly comparable. They presume, however, that the greater part of those vaccinated dwell in the more heavily infected areas in the country and of those of the control group, on the contrary, in the far less infected centres. The reduction of mortality would, therefore, in their opinion, be far from flattered and would actually be higher than  $\frac{2}{3}$ rds. This supposition may be right but it is highly possible that, in the heavily infected villages, many people abstained from vaccination, the more so since at times vaccination was available only at great distances from their dwellings. Considering that the 122 plague cases mentioned above concern only 85 of the 1,555 scattered villages, it would have been preferable if the comparative calculation of these cases had been confined to the population of the infected 85 villages only. Even then, however, exactly comparable mortality rates would not have been guaranteed, in case many families or even entire villages might have refrained from vaccination and in case the epidemic might not spread diffusely but was rather localized. Anyhow Girard and Robic are convinced that they achieved far better results with this live vaccine than with several dead vaccines in former years, which always yielded disappointing effects in test animals as well as in man.

The general vaccination of the population was here started in the last week of January 1935 in four centres of the Preanger with four squads at a time,

consisting of a native physician and four qualified nurses (mantris). At each centre the number of people daily vaccinated averaged 2,500 and if feasible, in each village, vaccination was completed in one day. Beginning in those sub-districts where plague had claimed the greatest number of victims, almost the whole area of the Preanger in which plague prevailed, comprising about 2 million inhabitants, was vaccinated in the course of the year 1935. At the end of November, when vaccination had to be discontinued on account of the Mohammedan fasting month (Ramzan), the number vaccinated amounted to round about 1,875,000, comprising more than 94 per cent of the population. Besides re-vaccination was started in some villages in which plague showed an increase after a lapse of time; to this re-vaccination also more than 90 per cent of the population submitted. Moreover, in October vaccination was carried out in some few foci of plague left in Mid-Java. The total number of vaccinations in 1935 amounted to 2,082,281, that of re-vaccinations to 236,056.

As for the results of this general vaccination it is impossible to estimate the reduction of mortality attained, due to the lack of exactly comparable controls; this can, however, be approximated by comparing the mortality rate of those vaccinated with that of those not vaccinated. The latter, comprising round about 10 per cent of the population, as a rule were absent not because of their being unwilling to submit to vaccination but because they were elsewhere engaged (market, planting, funeral, etc.); the non-appearance of an entire family occurred only exceptionally. The mode of living of those vaccinated and unvaccinated, was in the main exactly the same and an equal risk of infection may, therefore, be presupposed for both groups that differ only in numerical strength.

Supposing that those vaccinated comprise 9/10ths of the whole population, it is clear that a reduction of the mortality rate to 10 per cent means a proportion between the number of cases of bubonic plague among those not vaccinated to that among vaccinated of 1: 0.9, a reduction to 15 per cent a proportion of 1: 1.35, to 20 per cent of 1: 1.8, to 25 per cent of 1: 2.25 and so on. A proportion of the number of deaths of 1: 9 would mean an equal mortality in the two groups, due to inefficacy of the vaccine or to complete loss of immunity. In the 24 sub-districts of the Regency of Bandoeng which had been successively vaccinated, 3,990 cases of plague were recorded in 1935: 2,680 before or during vaccination, 96 in the first week after vaccination (of which 51 had been vaccinated) and 1,214 more than 7 days after vaccination, viz., 475 among unvaccinated\* and 739 among vaccinated. The proportion of the number of deaths in the two groups was 1: 1.55, which means a reduction of the mortality in vaccinated to 17 per cent.

This reduction fairly approaches that attained in the preliminary alternating test vaccination in the sub-district Batoedjadar (10 per cent), considering that primary pneumonic plague is included. The difference can be further explained by the rather rough manner in which this percentage is calculated, seeing that the percentage of vaccinated, averaging 90, ranged between more than 95 and hardly 80 in different villages. In a village with a percentage vaccination of 95 and

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\* Those not re-vaccinated have been ranged among the unvaccinated, when re-vaccination has taken place in their village.

a reduction of mortality to 10 per cent the proportion between the number of deaths in vaccinated and unvaccinated is not 1 : 0·9 but 1 : 1·9, in a village with a percentage vaccination of 80 it is 1 : 0·4. When, however, calculated on the base of a 90 percentage vaccination mortality would be in the first case reduced to only 21 per cent, thus amounting to twice the actual mortality, in the latter case to 5·5 per cent, i.e., to half of the actual mortality. So it is clear that with a calculation of the reduction on the base of an average vaccination percentage of 90, results will be flattered in those villages where this percentage is lower and minimized in those where it is higher. This drawback might be partly obviated by the determination of the number of deaths in each village separately on the base of the actual percentage of vaccinated but, apart from the figures growing too small, even then exactly reliable results would not be guaranteed, since the percentage vaccination of the accessory hamlets of one and the same village widely differs so that through the localized character of plague this average percentage cannot give a satisfactory correction neither. By the above calculation I, therefore, merely endeavoured to make it plausible that the favourable results of the preliminary test vaccination (the statistical value of which is beyond doubt) are also attainable in mass-vaccination.

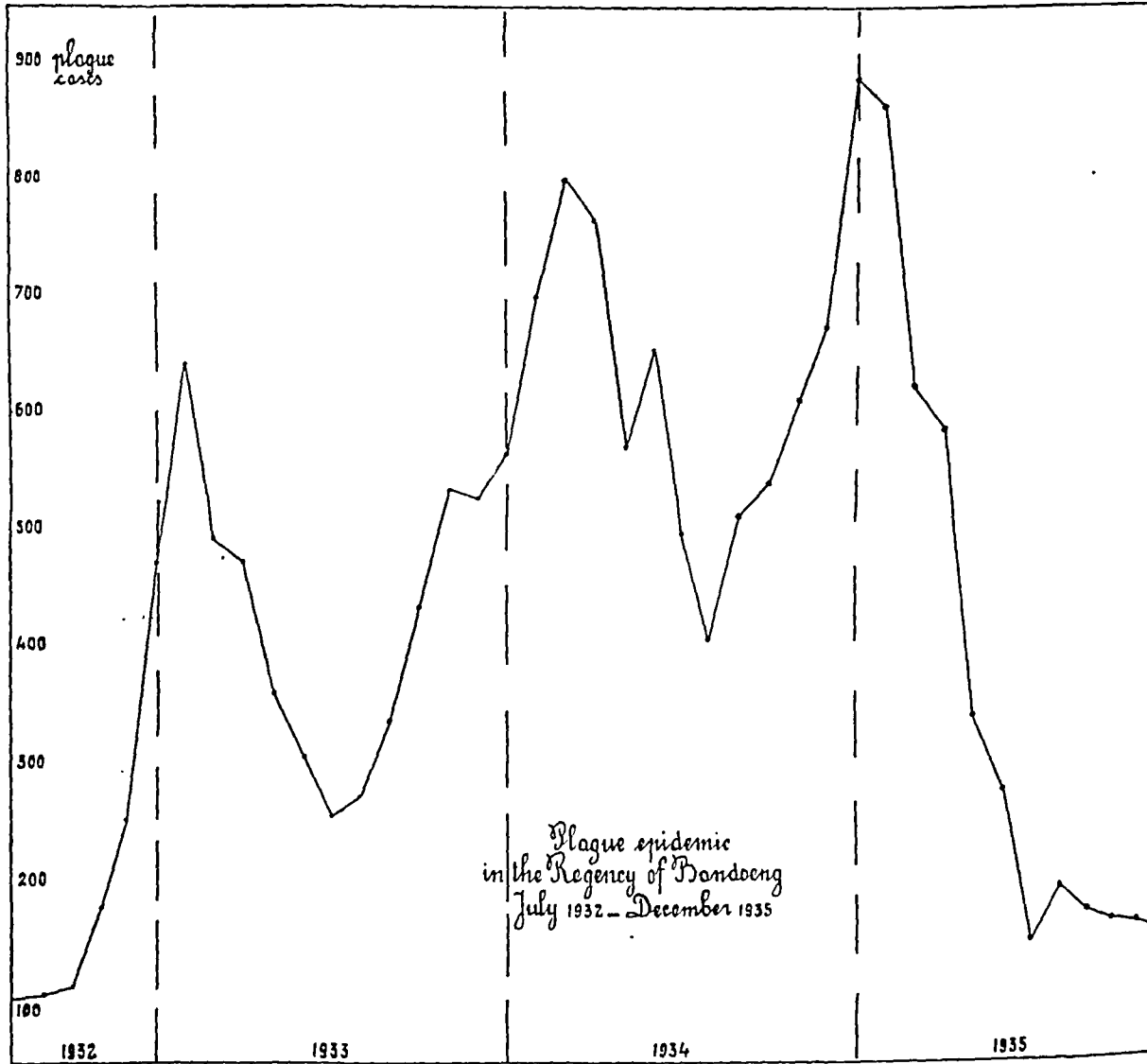
This conclusion is further substantiated by the suggestive course of the plague epidemic in the Regency of Bandoeng as compared with that of the last years. The incidence of plague in this area shows a typical, remittent character exactly corresponding to that of all Java as already described in many other papers: an increase at the beginning of the third quarter, the driest season, with its acme in the months round about the end of the year and a decrease half-way through the wet monsoon with the minimum in the middle of the year. While in the years 1932-1933, 1933-1934 and 1934-1935 this rise was regularly noted with an increasingly higher peak and smaller decline, in 1935 not only a quicker and steeper decline occurred but the curve was maintained on this low level for months and not until December was a slight rise noted (*vide* Graph). In connection with this rise stress must be laid upon the fact that during this month, in which the Mohammedan Ramzan occurred, re-vaccination was almost stopped; besides there prevailed many small outbreaks of primary pneumonic plague, against which vaccination is powerless.

Anyhow, the results obtained may be considered as rather satisfactory, since the vaccination as a plague repressive measure answers the aim in view, viz., the saving of human lives. Emphasis, however, must be laid on the difference between bubonic plague and other infectious diseases. In the latter, spread through contact from person to person, immunization of man may so deprive the infecting agents of their suitable medium as to cause these diseases to decrease even to vanishing point. In plague, however, not man but the rat is the originating source, human plague is only secondary to rat plague, which spreads undisturbedly despite immunization of man and is liable to prey on an increasingly greater number of victims as soon as immunity is on the wane. Though immunity may be restored by re-vaccination, this measure will, in the course of time, fail to guarantee success because of people being fain to abstain in increasingly greater numbers from periodical re-vaccination. Great advantage as the vaccination with a live vaccine in the repression of plague in man may be, the improvement of housing conditions remains indispensable and must be maintained as the only rational preventive



method capable of affecting the living conditions of the rat and of eradicating rat plague, the root of this evil.

GRAPH.



#### MICROBIC DISSOCIATION AND LOSS OF VIRULENCE.

The behaviour of the strain 'Tjiwidej' draws attention to loss of virulence under cultural conditions, a phenomenon characteristic of all pathogenic bacteria and already repeatedly described in connection with *Bacillus pestis*. During the first epidemic in Hongkong Yersin (1894) already established the fact that the

plague bacillus may easily lose its virulence: observing its growth on peptone-agar he noted that, after a few days, some colonies characterized by their size and higher rate of growth, when sub-cultured, appeared to have but slight if any virulence left for guinea-pigs. In connection with these findings Yersin suggested the possibility of immunization with such strains.

This experience of Yersin closely touches the problem of microbic dissociation. Until recently, however, according to general opinion, a culture was considered to consist of organisms all equally virulent and all of which shared equally in decrease and loss of virulence. It was not before 1927 that Hadley, the protagonist of microbic dissociation, opposed this conception in a general sense and threw full light on this problem, referring to numerous investigations: in the first place to those of de Kruif (1921, 1922) in *B. lepi-septicus*, one of the members of the *Pasteurella* group. De Kruif distinguished two types of colonies and stated that virulence was associated with the normal or 'smooth' type and not or only in a slight degree with the abnormal or 'rough' type. The scarcer the smooth type and the more preponderant the rough, the greater was the decrease of virulence, which was entirely lost as soon as the culture had become entirely of the rough type.

Pirie (1929) in an exact investigation into the dissociation phenomenon was the first to prove that these characteristics were also demonstrable in plague bacilli. In a sub-culture of a 2½ year old strain he distinguished, along with the normal type, designated as 'smooth', another type marked as 'rough'. The former appeared to be rather virulent, the latter absolutely devoid of virulence for rats and guinea-pigs. Contrary to de Kruif, who found that vaccination with the avirulent rough type of *B. lepi-septicus* was able to protect test animals against subsequent infection with a virulent strain (one experiment with two rabbits only!), Pirie's experiments with the rough type of *B. pestis* yielded a negative result, so that he was inclined to deny all immunizing value to this variant.

Bessonowa and Lenskaja (1931) found, among 150 plague strains, a few giving a diffuse turbidity when growing in broth. Seeded on agar-medium one of them showed a form of colony distinctly different from the normal type, also available on the same plate. The normal type was of the same virulence as the original strain, whereas the variant with its atypical growth appeared to be avirulent. In another strain, also giving diffuse turbidity in broth, the normal appearance of the colony was maintained and its virulence did not stand behind that of the original culture. In the opinion of Bessonowa and Lenskaja (*loc. cit.*) this atypical variant must be considered as the smooth type and the typical one—with agglutinative growth in broth—as rough; thus in plague bacilli (just as in *B. anthracis*) not the smooth but the rough type would be associated with virulence.

Though this contention agrees with the conception adhered to by Hadley (1927), I must oppose it. Arkwright (1921), who was the first to make these distinctions, based this denomination on the coli-typhoid-dysentery group in which the normal, freshly isolated virulent type is indeed characterized by growth in round, smooth and shining colonies, while the colonies of the abnormal type, which has little or no virulence, are conspicuous by their irregular form and dim and rough surface. Arkwright rightly designated the normal, original type as 'smooth'

and the abnormal variant as 'rough'. This, however, does not imply that this purely morphological distinction is applicable to all species of bacteria. On the contrary there are several bacteria giving rise to different types which cannot be distinguished on the base of this classification. To these species also belongs the *B. pestis*, whose normal growth departs from the usual one and which, contrary to most bacteria, shows no homogeneous turbidity, when grown in broth. However, it is illogical to designate the normal virulent type as a rough variant, which has nothing in common with it and, on the contrary, is characterized by the loss of many biochemical and antigenic properties.

This morphological distinction must, obviously, give rise to confusion, when generally applied. The more so since the cultural behaviour of many pathogenic bacteria—especially of the *B. pestis*—largely depends on the nature of the culture medium. Apart from the undeniable influence of different factors such as the peptone percentage, the degree of humidity, the pH of the medium, etc., many other circumstances unknown at present may also play a part. That is the weak point in the conception of the relation between bacterial cyclogeny and microbic dissociation as presumed by Hadley, based as it is almost exclusively upon cultural conditions, often so unfavourable to the organisms that many of the alterations observed must be considered as symptoms of atrophy and degeneration and must be ascribed to mutilation rather than to mutation, as is so aptly observed by van Loghem (1929, 1930).

That confusion already prevails is clear from the fact that according to Pirie (1929) the normal virulent type of the plague bacillus, characterized by colonies he called 'smooth', gives turbidity when grown in broth, whereas Bessonowa and Lenskaja (*loc. cit.*), on the contrary, consider—rightly—growth in broth without turbidity as normal but designate the colonies of this virulent type as 'rough'. The aspect of the smooth and the rough type as shown by their photographs exactly agree with the types observed by me, with the reservation that the normal type, growing in broth without causing turbidity and agreeing with their rough type, is called 'smooth' by me and conversely their 'smooth' is 'rough' with me. Burgess (1930) confines himself in the discussion of this phenomenon to a morphological description of the colonies. He distinguishes 3 types: small colonies with a fringe—the normal type—small round colonies with only a little, if any, fringe and larger colonies with irregular form. Vedder's (1932) classification is also based on such distinctions; his F, G and D types of colonies of *B. pestis* and pseudotuberculosis, however, do not exactly answer to the description of Burgess and even less to those of Bessonowa and Lenskaja.

Anyhow, the most important conclusion to be drawn from these divergencies is that the degree of virulence—whatever relation to antigenic structure may exist—cannot be accounted for by the morphology of the colony. Whereas Pirie stated a marked difference of virulence between his two types, Burgess' experience with his 3 types was not suggestive of such a differentiation and according to Bessonowa and Lenskaja atypical growth was associated with loss as well as with maintenance of virulence. By my investigations, too, no direct parallelism between morphology and virulence was established: both variants of the strain 'Tjiwidej' were absolutely avirulent notwithstanding that the type, which possessed highly antigenic potency, did not differ as to its growth in any respect from a typical virulent strain.

That the number of possible variants is not exhausted with those mentioned here, I experienced to my disadvantage. Returning from my Home leave in the middle of 1934 I continued my investigations with the strain 'Tjiwidej', using the sample taken with me to Europe and kept in stab-culture, but without any further precautions. It was the first time since 1930, the year I started my experiments with this strain, that it failed me: the only explanation left was a further dissociation in this strain. I had indeed observed some atypical colonies on the plate, small round colonies without the typical fringe, and two additional series of rats were, therefore, inoculated, one with a vaccine prepared with the apparently unchanged typical variant, the other with that prepared with the atypical variant. Little did I imagine that the typical variant, which showed no morphological alteration whatever, could have entirely lost its antigenic potency. I then appealed to the original strain preserved in stab-culture and uninterruptedly kept in the cold storage at Bandoeng. A comparative experiment (inoculation with 1/50 agar-culture, infection as usual) showed undeniably that the immunizing properties of the original strain had remained unaffected.

TABLE VIII.

Type.	RAT.		GUINEA-PIG.	
	Vaccinated.	Controls.	Vaccinated.	Controls.
1. Tjiw. sm. Europe ..	10 : 0/10	} 10 : 0/10	10 : 6/4	10 : 1/9
Pure typical variant ..	10 : 0/10		..	..
Pure atypical variant	10 : 2/8		..	..
2. Tjiw. sm. Europe ..	10 : 0/10	} 10 : 0/10 {	10 : 7/3	} 10 : 0/10
„ „ Bandoeng ..	10 : 10/0		10 : 10/0	

As soon as the manufacture of the live vaccine, produced to the amount of at least 50,000 doses weekly, was no longer a matter of difficulty, I more closely examined the problem of spontaneous loss of virulence in a number of plague strains from 5 to more than 15 years old and stored at 5°C. in stab-agar-culture after receipt or after isolation here. The strains concerned are those that were used in my differential diagnostic research of the *Pasteurella* group in 1924-1925 in addition to those which had been isolated from different areas of the Preanger in subsequent years. Next to the strains 'Tjiwidej' and 'Java' I experimented upon 12 other strains available from different places: the strains 'Maasland' and 'Sadko' isolated from rat plague on board of ships in Amsterdam in 1920, the strain 'Smyrna' (1918) obtained from Kral's museum, the strain 'Dupré' from the epidemic in Paris (1920), the strain 'Bombay humana' and 'Bombay rat' (1919) from Bombay, the strain 'Harbin' from the second pneumonic plague epidemic in Manchuria (1920-1921), the strain 'Medan' from the small epidemic on the East Coast of Sumatra (1918) and four more strains obtained locally from the Preanger in the period 1925-1929.

As a preliminary experiment all these strains were first sub-cultured in broth and one guinea-pig was inoculated with 1 c.c. of this culture of each strain; if this infecting dose caused no ill effect whatever, a second guinea-pig was injected with a whole agar-culture. If even this dose appeared harmless, the whole stab-culture was cut to pieces each of which was seeded in a broth tube; after sub-cultivation of each of these tubes on agar a full agar-culture of these sub-cultures was each inoculated in a guinea-pig. By this proceeding I succeeded in proving that the strain 'Medan' was still virulent, notwithstanding that the same dose of a full agar-culture derived from the stab-culture in the usual way (after seeding of a loopful in broth) had yielded a negative result. Even more striking was the finding that the original strain 'Tjiwidej', preserved in stab-culture in September 1929 after rat sub-passage and which since January 1930 had yielded absolutely negative results in many animal tests, appeared to be still virulent.

Of the 14 strains investigated in this way, 11 appeared still to be virulent, 8 already after the first inoculation of 1 c.c. of the broth culture, 3 after injection of an entire agar-culture ('Smyrna', 'Medan', 'Tjiwidej', the last two only after exhaustion of the whole stab-culture), regaining their original degree of virulence after some few animal passages. Three strains ('Sadko', 'Dupré' and 'Java', of the last of which the original culture appeared meanwhile to have vanished) had become absolutely avirulent, viz., a dose of a whole agar-culture subcutaneously injected into guinea-pigs caused no symptoms at all (buboes, etc.). Following Kolle and Otto (*loc. cit.*) and Strong (*loc. cit.*), I chose a whole agar-culture as maximum dose, as these authors consider a strain as suitable for human vaccination only if the subcutaneous injection of guinea-pigs at a dose of 2 agar-cultures is not fatal; afterwards Strong (1908*a* and *b*) reduced the dose limit to 1 agar-culture. The condition on which the choice of this dose is based, in my opinion, is insufficient, not so much because of its being arbitrary but especially because death and not the presence of symptoms is considered the criterion of the noxiousness of a vaccine. I myself should not allow a strain to be used for immunizing purposes, which can give rise to buboes in guinea-pigs notwithstanding the fact that a whole agar-culture is not able to kill them, a not exceptional experience. Conversely, I should not be opposed to a strain that is liable, in larger doses, to kill test animals from toxæmia; even a dead vaccine is apt to do so. The Haffkine vaccine in a dose of only  $\frac{1}{4}$  c.c. gives a percentage mortality of 20 to 30 in rats from acute intoxication; this was, however, never considered a contra-indication to the vaccination of man with a dose of 3 c.c. to 5 c.c. Each strain, even 'Tjiwidej', retains enough of its toxicity to remain fatal to test animals if not from subcutaneous anyhow from intraperitoneal injection. For convenience' sake, however, I adhered to the dose of 1 agar-culture as a comparative base of qualifying strains avirulent on the condition that buboes were absent in test animals.

Thereafter I investigated the question as to whether avirulent variants could be isolated from those strains, which had retained their virulence. As regards the markedly attenuated strains 'Medan' and 'Smyrna', this seemed rather probable in connection with the behaviour of the strain 'Tjiwidej', but for the other strains this remained an open question. To solve this problem a broth-culture of each of these strains was diluted to such a degree as to obtain well-isolated colonies when one drop was smeared on a blood-agar plate. After 48 to 72 hours

some 10 colonies were sub-cultured in broth and from broth on agar. Finally 10 guinea-pigs were inoculated with a full agar-culture, each originating from one colony and presumably, for the greater part if not all, from one bacillus of the same strain. When a guinea-pig remained unaffected, 10 more guinea-pigs were inoculated each with an entire agar-culture, originating from the same avirulent colony, in order to make sure that a really avirulent variant had indeed been secured. If none of the guinea-pigs survived, the experiment was repeated with 10 other freshly isolated colonies. Finally, the survivors of these experiments were infected in the usual way quickly to obtain an impression of the antigenic value of the strain concerned. The results of these experiments are collected in Table IX:—

TABLE IX.

STRAIN.			Virulence (+ = vir.) (- = avir.)	Infection with 10 different colonies (full agar- culture).	INFECTION.		Infection with test dose.
Number.	Name (Date of isolation).				With 10 other colonies (full agar- culture).	With one same aviru- lent colony (full agar- culture).	
I	Maasland 1920	+	10 : 0/10 <sub>5-8</sub>	10 : 0/10 <sub>5-8</sub>	..	..	
II	Sadko .. 1920	-	..	..	..	..	
IV	Smyrna .. 1918	+	10 : 9/1 <sub>13</sub>	..	10 : 10/0	19 : 3/16	
VI	Dupré .. 1920	-	..	..	..	..	
VII	Bombay humana 1919	+	10 : 1/9 <sub>5-6</sub>	..	(10) 9 : 8/1 i.c.	10 : 9/1	
VIII	Bombay rat 1919	+	10 : 7/3 <sub>4-6</sub>	..	10 : 10/0	17 : 16/1 i.c.	
X	Harbin .. 1920-1921	+	10 : 0/10 <sub>3-6</sub>	10 : 0/10 <sub>3-6</sub>	..	..	
XI	Java (1920) 1926	-	..	..	..	..	
XII	Medan .. 1918	+	10 : 10/0	..	10 : 7/3*	17 : 17/0	
XIII	Preanger 1925	+	10 : 0/10 <sub>4-7</sub>	10 : 0/10 <sub>5-6</sub>	..	..	
XIV	Tasikmalaja 1926	+	9 : 0/9 <sub>3-4</sub>	10 : 0/10 <sub>3-4</sub>	..	..	
XV	Bandoeng 1929	+	10 : 8/2 <sub>6-8</sub>	..	10 : 10/0	18 : 17/1	
XVI	Soemedang 1929	+	10 : 1/9 <sub>3-5</sub>	..	10 : 10/0	(11) 10 : 9/1	
XVII	Tjiwidej .. 1929	+	10 : 10/0	..	..	10 : 7/3	
Controls ..		..	..	..	..	10 : 0/10	

\* = toxic death; i.c. = intercurrent death.

The subscript figures indicate the average number of days of survival after infection.

From this it appears that next to the strain 'Tjiwidej' not less than 6 out of 10 virulent strains gave rise to avirulent variants; these strains do not all belong to markedly attenuated strains such as 'Smyrna' and 'Medan', but among them are 4 out of 8 strains whose virulence was apparently intact. Of the last group, in two ('Bombay rat' and 'Bandoeng') the greater part of the bacilli (7 and 8 respectively out of 10 colonies) was avirulent, of both the others, however, only 1/10th (1 out of 10 colonies). The possibility, suggested by the seemingly avirulent parent cultures of the strains 'Tjiwidej' and 'Medan' which appeared actually to have conserved some virulence, by closer investigation was substantially in agreement with Hadley's contention that in many, if not in all, pathogenic bacteria highly virulent and avirulent organisms may be available in the same culture\*. This explains the sudden return to virulence of an apparently avirulent culture so often observed, which is actually due to the fact that not all organisms of the culture concerned were avirulent. Only a culture originating from one avirulent bacillus (i.e., colony) is absolutely avirulent and the chance of its return to virulence may be considered as excluded.

This dissociation, now well established, is the more striking since no morphological differences between the colonies can be detected: all the colonies show the typical aspect of a small hemisphere with an irregular fringed zone. In 4 strains ('Maasland', 'Harbin', 'Preanger' and 'Tasikmalaja') no dissociation could be established by this method after repeated experiments; this does not, however, exclude that no dissociation existed, since the examination of hundreds of colonies would be required to prove an absolutely negative result. The experience that this dissociation occurs in some strains within some months and is absent in others for long years, must be accounted for by the individual behaviour of bacteria. Owing to our relatively rough biochemical and serological methods the essential of these intracolonial changes will probably remain inaccessible to us.

According to the results obtained by infection of the survivors (Table IX, last column) the immunizing potency of all but the strain 'Smyrna' was rather satisfactory. Since the vaccinating dose amounted to an entire agar-culture I made a series of experiments in rats and guinea-pigs with all 10 avirulent strains—of which 7 were variants of virulent parent cultures—hoping to obtain an even better antigen than the strain 'Tjiwidej'. A comparative experiment with the strain 'E. V.' from Madagascar, that was kindly sent to me by Dr. Girard, was also made. The vaccination was performed as usual with one injection of 1/100th agar-culture, followed by infection with the usual test dose (in guinea-pigs a ten-fold dose) 3 weeks later. The results of these experiments (4 series) are collected in Table X. As not all strains were used in every series, in the last 2 columns the results of the comparative experiments in rats and guinea-pigs with the strain 'Tjiwidej' are mentioned; these are exactly comparable with those yielded by the strains according to the first two columns.

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\* Some 9 more strains have since been examined, isolated from virulent plague matter (6 from blood of plague patients, 3 from rat plague) and preserved in stab-culture. In not less than 6 of them I succeeded in ascertaining avirulent variants in the still highly virulent parent culture; the oldest of them had been isolated 3½ years before, the youngest six months only.

TABLE X.

STRAIN.		RAT.		GUINEA-PIG.		STRAIN 'TJIWIDEJ.			
						RAT.		GUINEA-PIG.	
No.	Name.	Results.	Percentage survival.	Results.	Percentage survival.	Results.	Percentage survival.	Results.	Percentage survival.
II	Sadko ..	(10) 10 : 1/9	10	(10) 10 : 0/10	0	10 : 4/6	40	10 : 8/2	80
IV	Smyrna ..	(10) 10 : 1/9	10	(10) 10 : 0/10	0	10 : 4/6	40	10 : 8/2	80
V	Dupré ..	(20) 20 : 10/10	50	(20) 20 : 8/12	40	20 : 13/7	65	20 : 18/2	90
VII	Bombay humana	(30) 30 : 21/9	70	(30) 30 : 30/0	100	30 : 19/11	63	29 : 27/2	93
VIII	Bombay rat	(30) 30 : 22/8	73	(30) 30 : 30/0	100	30 : 22/8	73	30 : 27/3	90
XI	Java ..	(20) 20 : 12/8	60	(20) 20 : 20/0	100	20 : 15/5	75	19 : 19/0	100
XIa	Java (toxic)	(40) 40 : 29/11	72.5	(40*) 36 : 36/0	100	40 : 28/12	70	39 : 36/3	92
XII	Medan ..	(20) 20 : 2/18	10	(20) 20 : 9/11	45	20 : 13/7	65	20 : 18/2	90
XV	Bandoeng ..	(20) 20 : 5/15	25	(20) 20 : 17/3	85	20 : 13/7	65	20 : 18/2	90
XVI	Soemedang	(30) 30 : 15/15	50	(30) 30 : 30/0	100	30 : 22/8	73	30 : 27/3	90
XVIII	Madagascar	(30) 30 : 15/15	50	(30) 29 : 29/0	100	30 : 24/6	80	29 : 28/1	96.5
XVII	Tjiwidej ..	(40) 40 : 28/12	70	(40) 39 : 36/3	92	..	..	..	..
	Controls ..	(40) 40 : 1/39	2.5	(40) 40 : 0/40	0	..	..	..	..

\* 4 toxic deaths.

From these figures it will be clear that two of these strains ('Sadko' and 'Smyrna') are absolutely worthless, and two ('Dupré' and 'Medan') possess only slight antigenic potency; the remaining strains have satisfactory immunizing value, though it sometimes markedly differs in rats and guinea-pigs. This is most striking as to the strain 'Bandoeng' with which 25 per cent of the rats survived against 85 of the guinea-pigs; especially in the first series the difference was striking: 70 per cent mortality in rats while the guinea-pigs all survived. In the strains 'Soemedang' and 'Madagascar', yielding exactly the same results, the difference is also marked and it is somewhat less pronounced as regards 'Java'. The strains 'Bombay humana', 'Bombay rat' and 'Tjiwidej' yielded the best results; it must be taken into consideration, however, that the survival rate obtained with the strain 'Tjiwidej' in these 4 series is below that of the average



rate of 80 per cent reached in all experiments with a vaccinating dose of 1/100th culture (*cf.* Table II); in two of the four series the percentage mortality was not less than 60 and 40 respectively, whereas a mortality rate of 40 was seen only once in the former extensive series of experiments. I, therefore, assume that the antigenic value of the strain 'Tjiwidej' in rats compares favourably with that of both the Bombay strains, though this does not appear from this small number of experiments.

As regards guinea-pigs, the strain 'Tjiwidej' must, however, be put behind them, not so much on account of the slightly differing mortality rates as on account of the incidence of symptoms of illness observed in survivors. While almost all guinea-pigs immunized with the strain 'Tjiwidej' showed distinct, sometimes even large buboes after infection, these were absolutely absent in those immunized with the Bombay strains, just as in those immunized with the strains 'Java', 'Madagascar' and 'Soemedang' and less pronounced in those immunized with the strain 'Bandoeng'. In agreement herewith the temperature of the 'Tjiwidej' guinea-pigs in the first days after infection rises to a somewhat higher level (39°C.) than that of animals inoculated with the other strains compared here, remaining normal (38.5°C.) against 40°C. and higher in controls. Apart from this, the 'Tjiwidej' guinea-pigs seem absolutely healthy and the impression imposes itself that the few failures, as far as they are not due to secondary pneumonic plague, are due to some intercurrent disease, even when post-mortem examination proves the presence of plague bacilli in the spleen.

A rather remarkable variant is the strain 'Java toxic' which, contrary to the strain 'Java', was not isolated from the original strain (which was lost in the meantime) but from its oldest sub-culture available since 1926. This variant, when subcutaneously inoculated, was occasionally fatal to guinea-pigs, not only with large doses but even with a dose of 1/100th agar-culture, post-mortem examination showing the typical aspect of plague though without buboes. In smears of the spleen, as a rule studded with necrotic spots, bacilli are scarce but cultures of spleen matter always give a positive result. When some 10 guinea-pigs are inoculated with such a sub-culture the effect is invariably the same: all with a rare exception, survive and the typical symptoms of acute plague are never observed no matter the number of sub-passages and the dose. Though the symptoms approximate those of death from toxæmia, it is curious that they manifest themselves rarely after 2 days but mainly as late as 5 to 7 days or more after the injection. Besides this variant, even in a dose of a whole culture, does not cause death in rats which are far more susceptible to the toxic action of the plague bacillus. Perhaps this suggests the possibility of the existence of an intermediate form which, though devoid of virulence, has not yet completely become saprophyte. If plague bacilli are apt to multiply for some time in the organism despite gradual destruction, it is conceivable that an increasing amount of toxic products may act upon the most susceptible among guinea-pigs.

Six to eight hours after a subcutaneous inoculation of his avirulent strain Strong (1908*a* and *b*) found numerous bacilli still viable at the place of injection, but usually no longer reclaimable in cultures after 24 hours. Strong, therefore, contends that the process of immunization occurs as a true vaccination, the organisms multiplying in the tissues for probably over 100 and more generations

and its successive groups of receptors stimulating the production of the corresponding groups of amboceptors in the animal body. It, therefore, might not be difficult to understand why immunity derived from vaccination in plague is higher than that obtained from the injection of dead organisms. From my experiments with the strain 'Tjiwidej', it appeared that bacilli were present in the tissues by far longer than 24 hours and reclaimable in cultures from the site of injection as well as from the organs especially from the spleen as late as 7 days after a subcutaneous injection. I do not believe, however, that this is due to an active increase of the organisms and that this accounts for the fact that a live vaccine compares favourably with a dead one. This difference, in my opinion, can easily be explained by the antigenic properties being intact in a live vaccine contrary to a dead vaccine artificially killed by heat or by the addition of some disinfectant, both of which are deleterious to them\*.

Anyhow, active increase in the animal body of the strain 'Java toxic', concerned here, seemed probable and I, consequently, tried to prove its presence in some of the guinea-pigs dead from an injection of a whole agar-culture (round about 5,000 millions viable bacilli). When bacilli were not too scarce in smears of the spleen, spleen matter was sub-cultured on blood agar plates in order to estimate the number of viable organisms with the dilution method already described. In two cases of death 5 and 11 days after the injection their number amounted to 500 and 1,500 millions respectively; of a third guinea-pig, dead after 7 days, the spleen contained more than 5,000 millions as a proof of increase of the bacilli. As a matter of course the possibility that all organisms injected would have been transported to the spleen and remained there intact for a week may be excluded; besides, the liver, as a rule, shows the same typical aspect with sporadic bacilli in its smears, so that in this organ too rather a large number of bacilli is presumably present.

Apart from this strain 'Java toxic' the above series of experiments shows that there are several avirulent strains of great immunizing potency, characterized, however, by differential results in rats and guinea-pigs. Most of these strains possess an antigen especially active in guinea-pigs but producing less or even slight effect in rats. The strain 'Tjiwidej', on the other hand, is the best antigen for rats, but must be put behind others as regards guinea-pigs. This difference is of a qualitative nature, as it cannot be overcome by increasing the dose.

This striking fact is of great practical significance for the vaccination of man, since a qualitatively different action of various plague antigens may be expected in man too. As it is not feasible to carry out comparative experiments with different antigens in order to establish how man stands as compared with the rat and the

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\* When the live vaccine is stored at 5°C. as standard suspension of 1/10th agar-culture per c.c., the number of viable bacilli remains unchanged for at least 4 weeks and its antigenic potency is fully maintained. Stored at room temperature (22°C.) the number of viable bacilli quickly decreases in the course of 7 to 14 days, though the immunizing properties remain almost intact. This suggests that spontaneous death of the bacilli is less noxious to the antigen, at least at the beginning, than their artificial death. The preservation of its full activity at a temperature of 5°C. is of great advantage as it allows of the manufacture of the vaccine being independent, to some extent at least, of the consumption. The preparation of the vaccine in its successive stages is now carried out weekly instead of daily (from stab-agar-culture in broth, from broth on a preliminary agar-culture, that—washed off in concentrated suspension—is used for the final cultivation). Though the preparation of a live vaccine is a matter of greater difficulties than that of a dead vaccine, we almost always had a sufficiency of such a stock as to be able to satisfy the demand without interruption.

guinea-pig, vaccination with a mixed vaccine, consisting of 2 or more strains, will guarantee the best results. As soon as amongst the strains which yield a survival rate of 100 per cent in guinea-pigs without any symptoms of illness one will be available, that gives as little reaction in man as the strain 'Tjiwidej', then vaccination will be carried on with a mixed vaccine. Such a vaccine will not present difficulties, its preparation being no matter of greater trouble than that of a single vaccine.

Finally, I would draw attention to the fact that, no matter the theoretical objections that may be advanced against the views on microbic dissociation, so ingeniously developed by Hadley, this phenomenon—when considered from a practical point of view—holds out the prospect of immunization with live vaccines against many epidemic infectious diseases. Just as this research led to a real vaccination against plague, it will prove possible when continuing the work of de Kruif (*loc. cit.*), White (1925), Balteanu (1926), Hadley (1931) and Felix and his collaborators (1934) to prepare absolutely innocuous live vaccines, in the first place against cholera, typhoid and bacillary dysentery, which will be of higher immunizing value than those now in use. As regards veterinary prophylaxis it is obvious that far better results should also be obtainable if this method be adhered to.

#### CONCLUSIONS.

1. Extensive series of experiments, carried out on wild rats and guinea-pigs with the live, and after some few months, spontaneously attenuated strain 'Tjiwidej', yielded results by far superior to those till now obtained with dead vaccine.

2. In further investigations with a number of strains 5 to 15 years old and preserved in stab-culture at a temperature of 5°C. it appeared that from several still virulent parent cultures avirulent variants could be isolated.

3. This loss of virulence occurs irrespective of the age of the culture and may or may not be associated with alteration in the form of the colony; in the former case the immunizing value, as a rule, is but slight.

4. Even when the typical growth is retained, these avirulent variants show great differences in antigenic potency as proved by animal test: several strains possess highly immunizing value and some appear almost entirely devoid of it.

5. The immunizing properties depend on a compound of antigens of which two at least could be demonstrated; one produces the highest degree of immunity in rats, the other in guinea-pigs. Besides this qualitative difference further quantitative differences obviously exist between these strains as to the partition of these two antigens.

6. From the results of a preliminary vaccination, in accordance with the alternating system, it appeared that mortality from bubonic plague in man can be reduced to 1/10th. The course of the epidemic after mass vaccination of a population of round about two millions also testifies to the value of vaccination as a plague repressive measure of high potency.

7. The use of a mixed vaccine, consisting of two or more strains of different efficacy in rats and guinea-pigs, promises even better results.

8. The presumption is justified that in other acute infectious diseases of an epidemic character the causative agents may also afford absolutely avirulent variants suitable for live vaccines superior to the dead ones in use up to this time.

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## THE STERILIZATION OF DRINKING WATER WITH MINIMAL DOSES OF CHLORINE.\*

BY

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### INTRODUCTORY.

THE Great War of 1914 to 1918 advanced the practice of chlorination of water-supplies to a remarkable degree and extension of the process has been so rapid in recent years in all parts of the world that it has come to be regarded as an essential measure in safeguarding water-supplies.

### DOSAGE OF CHLORINE.

The amount of chlorine required to kill bacteria in water is very small but considerably larger quantities have to be added owing to the demands made on the chlorine by the oxidizable organic and inorganic matter present in the water. Thus, the maintenance of 0.1 to 0.2 p.p.m. of residual chlorine after a twenty minutes' contact (detectable by the orthotolidine test) is considered to be the standard routine technique of chlorination, in order to ensure continuous action and effective destruction of the pathogenic bacteria. The determination and application of the correct dose is, therefore, of the utmost importance in rendering a water 'safe'. The test for residual chlorine after twenty minutes' contact is likewise considered to be the most accurate method of judging the efficacy of the treatment. The dose of chlorine for any water has, therefore, to be ascertained every day and in some extreme cases more often than once in twenty-four hours. There is, in addition, the question of tastes and odours, which develop in some waters, even when the correct optimum dose is added. In such cases, elaborate methods of de-chlorination

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have to be adopted or the tastes and odours have to be removed by special adsorbing or chemical agents. Finally, one has to contend with the vague fears—freely expressed by many engineers—that chlorinated water is responsible for rapid corrosion of the metal parts of engines and pumps and also of iron distribution mains. In 1926, we had one such complaint from a big municipal pumping plant.

#### PRELIMINARY EXPERIMENTS.

We, therefore, initiated certain experiments to ascertain if waters could be sterilized by doses of chlorine considerably short of the optimum dose as ascertained by the usual field test. We believed that, if successful, such doses would be acceptable both to the engineers and to the public.

Thirty-two samples including examples derived from wells, rivers, lakes, impounded surface waters, and the effluents from slow and rapid filters were tested. Graduated doses of chlorine, ranging from 0.05 p.p.m. to the actual ascertained optimum dose, were used in each case. The treated samples were then examined for the presence of lactose fermenters. We found that in nearly every case the water showed their absence in 60 c.c. with doses considerably smaller than the optimum. In 5 samples a dose of 0.05 p.p.m. was effective, in 11 cases 0.1 p.p.m. was effective, in 4 others 0.2 p.p.m. was similarly effective and in 10 others 0.3 p.p.m. effected sterilization. The ascertained optimum doses in these cases ranged between 0.5 and 1.5 p.p.m. Although the above results showed that much smaller doses than the ascertained optimum effected sterilization it was not possible to evolve a constant formula or factor that could be made to govern the exact relationship between the minimal dose and the ascertained optimum dose for all waters. As examples, we may give the case of a particular water with a chlorine demand of 1.5 p.p.m. showing the presence of lactose fermenters in 0.01 c.c. which was sterilized by a fifth of that dose, and of another water which required 1.1 p.p.m. by the field test and contained lactose fermenters in 10 c.c. but which was effectively sterilized by 0.05 p.p.m. Under these circumstances, it was not considered desirable to continue the experiments, as the results did not permit the adoption of a constant factor which could be used in the field for arriving at the proper minimal dose from the ascertained optimum dose.

#### VERDUNIZATION.

Our interest in this subject was, however, revived by the appearance in the *Journal of the American Water-works Association* of two important contributions from Nachtigall and Ali (1934) and Bunau-Varilla (1934) respectively, on the process called 'Verdunization'. In 1916, during the battle of Verdun, Colonel Bunau-Varilla, a French engineer in charge of water-supplies, succeeded in effectively chlorinating the water supplied to the French army with doses of chlorine about a tenth of the determined optimum doses and in producing an absolutely safe water free from any obnoxious taste or smell. This, he claims to have achieved by having recourse to violent agitation of the water immediately after the chlorine was applied in minimal doses. He later advanced the hypothesis that the sterilizing effect was due to the action of chlorine on the organic matter present in the water. It was no longer necessary to add sufficient chlorine to deal with the whole of the organic

matter present in the water. This method of Bunau-Varilla disregards the chlorine demand of the water and stresses the great importance of ensuring 'vigorous agitation' following the addition of chlorine. It is claimed for this method that clear waters from any source can be sterilized with 0.1 p.p.m. and slightly turbid waters from all sources with 0.2 p.p.m. of chlorine, provided vigorous agitation accompanies the addition of the chlorine. Verdunization is now reported to be in successful use in Paris, Lyons, Dieppe and many other cities in France, also in Brussels, Geneva, Lausanne, Genoa, Trieste, Seville and Lisbon, as well as in Algiers, Dakar, Saigon and Caracas.

Nachtigall and Ali (*loc. cit.*) do not, however, concede the above hypothesis of Bunau-Varilla, as in their experience (more or less similar to ours here in 1927), although several samples of water were sterilized with smaller doses than the optimum, there was apparently no uniformity in the relationship between the minimal and the optimum doses. They would, therefore, rely only on the orthodox method of applying the proper optimum doses, ensuring a residual of 0.1 to 0.2 p.p.m. of chlorine.

Bunau-Varilla (*loc. cit.*) criticizes the theoretical considerations relied upon by these two authors and adduces evidence based on what he calls 'three capital experiments' communicated to the French Academy of Sciences. These experiments would go to prove his contention that the 'destruction of the major part of the infectious bacteria is effected by ultra-violet rays emitted by the chemical action resulting from the contact between the organic matter of the water and the particles of chlorine distributed in the whole mass of water by the violent agitation to which it is submitted. The remaining bacteria are destroyed by the direct contact between them and the particles of chlorine which they happen to meet'.

Reiss and Techoueyres (1933) have explained the mechanism of the sterilization of water by 'Verdunization' by showing that when minimal doses of hypochlorite are added to the water there is a definite rise in the oxidation-reduction potential of the water, which is high enough to kill *B. coli* and pathogenic bacteria.

Imbeaux (1933) considers that the sterilizing power of chlorine and chlorine compounds varies with the oxidation potential. Bacterial reduction parallels oxidation potential (in volts), with chlorine, chloramine and azochloramide. Similarly, the addition of ammonia reduces both oxidation potential and sterilizing power of chlorine. Between 0°C. and 25°C. there is a gradual increase in sterilization and in oxidation potential. With increasing turbidity of the water, other factors remaining constant, there is a decrease.

Thresh *et al.* (1933) consider that the nascent oxygen hypothesis of chlorination is no longer tenable and that it is the chlorine that is *directly toxic* to bacteria by forming some compound with their substance and so affecting their physiological processes that life cannot continue. This view would appear to accord with the hypothesis of Bunau-Varilla in some respects.

Race (1918), however, states that though chlorine and its compounds are toxic to bacteria even in minute doses, the smaller concentrations produce merely a transient toxic effect which would permit the reproductive faculty of the organisms to be entirely regained.



Bunker (1935) casually mentions the fact that in one of the municipalities in Spanish America 'Verdunization' has proved a costly failure.

#### SECOND SET OF EXPERIMENTS.

We applied the Bunau-Varilla method to about sixty samples of water derived from various sources scattered over the Madras Presidency. No conscious selection was made of the samples which arrived in the usual course in the laboratory, and they are, therefore, representative of the typical drinking water sources in actual use. Both clear and turbid waters are included in this number. The technique employed was as follows:—

The theoretical optimum dose was ascertained in each case by the usual field test and the sample was divided into five portions of a litre each. Portion *A* received the optimum ascertained dose and was stirred with a glass-rod a dozen times and allowed to rest for twenty minutes. Portions *B* and *C* each received the minimal dose of 0.1 or 0.2 p.p.m. as the case might be, and *B* was stirred with a glass-rod a dozen times. *C* was taken in a stoppered bottle and vigorously shaken up for ten minutes by lateral movements allowing a foot excursion each time. It was then allowed to rest for ten minutes before being tested along with the others for lactose fermenters. A fourth portion *D* received no chlorine at all but was subjected to the same vigorous agitation in a bottle as *C* above. The object of this was to ascertain what effect the agitation alone, apart from the chlorine, had on the sample of water. Sample *E* was our control, i.e., the water as obtained from the source. All the samples, as detailed above, were inoculated into MacConkey's bile-salt lactose-broth tubes within half to one hour after they had been subjected to the respective treatments noted above. The organisms of the *coli* group present in samples *B* and *C*, which had probably been subjected only to a transient toxic action by the chlorine applied in minimal doses (*cf.* Race, *loc. cit.*), had thus ample opportunities of regaining their vitality in the enrichment medium in which they found themselves in less than an hour of their being acted upon by the chlorine.

Our results, however, show that the toxic action, even of minimal doses of chlorine, has been more permanent than transient. In fact, lactose fermenters were absent in 60 c.c. in all clear water samples in portions *A*, *B* and *C*. The optimum ascertained doses in these cases varied from 0.4 in a few through 0.7 in many to 1.3 in another few. The minimal dose for the clear water samples was a uniform 0.1 p.p.m. In the case of slightly or highly turbid waters, we had only 60 per cent success with 0.2 p.p.m. Some of our samples in this series were definitely discoloured and muddy or opaque. These were drawn from rivers or lakes containing flood water. Our failure to get as good results as in the case of clear waters was probably due to this fact.

The *D* portions of our series, i.e., those which had been subjected to a mere vigorous shaking without any chlorine added thereto, showed that they were bacteriologically very much worse than the control *E* and necessarily still more so than the *C* portions. Bacteria possibly present in the original sample in clumps had apparently got dispersed by the vigorous shaking and owing to this distribution were detectable in the broth tubes even in high dilutions. If this explanation is correct, the hypothesis of Bunau-Varilla, that the ultra-violet rays set up by the

chlorine act instantaneously on the bacteria, would appear to gain some measure of support from the above finding.

We have not been able, from the results obtained in this study, to establish any uniform relationship between the organic content of the waters tested and the ascertained optimum dose of chlorine. In the case of clear waters, however, showing low figures for organic matter (e.g., free ammonia, trace to 0.002; albuminoid ammonia, trace to 0.005; oxygen absorbed by potassium permanganate in 4 hours, Tidy's process, up to 0.040 parts per 100,000), the ascertained optimum dose was nearly always between 0.4 and 0.7 p.p.m. In the case of clear waters showing considerably higher figures for organic matter, this dose varied from 0.4 to 1.5 p.p.m. In the case of hazy and turbid waters, this dose varied similarly. The organic content of a water cannot, therefore, be the sole determining factor in efficient chlorination.

#### DISCUSSION.

It would, therefore, appear that the bacterial sterilization of drinking waters particularly of the clear waters from wells and filter plants, can be effected by using 0.1 p.p.m. of chlorine empirically. We do not subscribe to the hypothesis of vigorous agitation being the most important factor controlling the success or failure of the minimal dose. For, in the experiments reported above, both our *B* and *C* portions yielded practically identical results. The term 'violent or vigorous agitation' is rather vague, as no means of measuring the vigour or violence has been indicated by the sponsor of the method. As stated above, since both the *B* and *C* portions of our samples have behaved similarly in the enrichment medium, we are left in doubt as to whether our stirring a dozen times with a glass-rod produced sufficient vigour to induce the ultra-violet rays required for the sterilization of the *B* portions. We believe that in actual water-works practice the procedure would be to pass the water and the minimal dose together through a centrifugal pump (McConnel, 1935). In this case the actual contact of each particle with the chlorine *plus* agitation could probably be reckoned in seconds rather than in minutes. On this basis, the mere stirring with a glass-rod a dozen times might be agitation vigorous enough for the bactericidal action in the case of the *B* portions and it is only on that assumption that the Bunau-Varilla hypothesis could be considered to have been established in our experiments. Experiments on a small plant, using minimal doses and a mechanical agitator, are under way and should the results of those experiments point to the same conclusions, this method would doubtless go far to effect considerable economy in the chlorination of water-supplies in India, and would eliminate the troubles met with in the matter of tastes and odours resulting from the application of large doses of chlorine. There would also be no need for a laboratory or trained personnel to control the process of chlorination at each water-works.

#### CONCLUSIONS.

The results of the application of minimal doses of chlorine to 100 samples of water from different sources scattered over the Madras Presidency, show that *clear* waters from wells, galleries, and filter plants can be sterilized by amounts of chlorine which, in many cases, are from a fifth to a tenth of the optimum ascertained doses.

Should these results of our laboratory experiments be confirmed on a plant scale, the process would result in (i) effecting considerable economies in the chlorination of water-supplies, (ii) ensuring relative freedom from taste and odour troubles and (iii) dispensing with the need for laboratory control of the process from day to day.

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# A COMPARATIVE STUDY OF CULTURE AND ANIMAL INOCULATION AS METHODS FOR ISOLATING TUBERCLE BACILLI FROM PATHOLOGICAL MATERIALS.

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## INTRODUCTION.

IN the course of our work on the isolation of the tubercle bacillus from materials derived from pulmonary and other forms of tuberculosis (Ukil, 1930, 1933), we had to employ different methods for the purpose, viz., direct culture from uncontaminated materials, culture from contaminated materials after preliminary treatment with chemicals to destroy the secondary organisms, and culture after guinea-pig passage. The isolation of the tubercle bacillus from contaminated materials presents many difficulties. Isolation by animal passage is not only a slow method but is more costly. The price and cost of maintenance of two guinea-pigs, which we utilize for each material, for 8 weeks at Calcutta is approximately Rs. 2-8-0, whereas the cost of 6 tubes of any of the suitable media does not exceed an anna or 1/40th of the cost of the animals. Guinea-pig inoculation has long been widely used as a safe, sensitive and reliable method for isolating tubercle bacilli. While a small amount of material is required for cultures, comparatively large amounts (8 to 10 times) are required for animal inoculation. Culture methods would certainly have several advantages over animal inoculation, if it could be proved that they were as accurate and reliable as guinea-pig inoculation for indicating the presence of tubercle bacilli in a material. Apart from the question of cost, culture takes a shorter time for isolation when it can be utilized for typing even in a small laboratory and the method is free from any chances of vitiating the

findings by intercurrent infections or spontaneous tuberculosis developing in the animals. It would be worth while, therefore, to know how guinea-pig inoculation compared with culture as a method for isolating tubercle bacilli from pathological materials and whether the culture method could be substituted for animal inoculation.

The culture method has been employed recently in several quarters, resulting in the employment of improved media and technique of isolation. Egg has formed an essential constituent of many media devised by Dorset (1902), Lubenau (1907), Petroff (1915*a* and *b*), Loewenstein (1924, 1933), Sumoyoshi (1925), Hohn (1926), Petraghini (1927), Corper and Uyei (1928), and Woolley and Petrik (1931). The presence of egg-yolk has been considered to be more important than that of the white. The use or addition of potato as a favourable component of media has been advocated by Pawlowsky (1888), Corper and Uyei (1928), Calmette and others. Glycerine has been used in many media as a stimulant to the growth of the tubercle bacillus. The growth-promoting influence of certain vegetable extracts, such as vitamin B, starch, etc., has also been studied.

It has been stated (Corper and Uyei, 1928) that several thousand tubercle bacilli per c.c. (100,000 according to some) must be present in a material to enable them to be demonstrated by microscopic examination in smears. Many of them may not be in a living condition, while the presence of secondary organisms renders a preliminary treatment with chemical digestives or with dyes necessary. The preliminary treatment of materials with chemicals, such as antiformin (Uhlenhuth and Xylander, 1908), caustic soda (Petroff, *loc. cit.*) and sulphuric acid (Loewenstein, *loc. cit.*) as well as the use of dyes in the preparation of media, no doubt kills the secondary organisms but has the disadvantage of destroying thinly-walled tubercle bacilli, of retarding the growth of others and of 'denaturing' them. The strength of the solution and the duration of treatment by the acid or alkali are important factors to be considered in this connection. The optimum dilution of the solution and the period of digestion have, therefore, got to be found out. The behaviour of certain strains towards these chemicals has to be considered as well. Petroff (Malkani, 1929) and others have shown that 'S' colonies develop better on alkaline media and 'R' colonies on acid media. In the hope that 'S' colonies could be recovered better by alkaline digestion and 'R' colonies by acid digestion, we employed both these methods of digestion in our culture work in the initial stages. The addition of dyes, such as gentian violet, basic fuchsin, crystal violet, brilliant green and malachite green, etc., also inhibit the growth of tubercle bacilli. Attempts have been made by Loewenstein (1924), Sumoyoshi (*loc. cit.*), Corper and Uyei (1928), Sweaney and Evanoff (1928) and other workers to overcome these drawbacks by improving the culture medium.

#### METHODS OF CULTURE EMPLOYED.

In culturing tubercle bacilli from pathological materials, the subject has to be considered from three standpoints, namely, the treatment of the material, the culture medium and the inhibiting substance.

*Treatment of the material.*—In case of materials, such as pus from cold abscess, serous fluid, etc., we first tested them for the presence of secondary organisms

by inoculating broth and agar media and incubating for 24 hours. If no contaminating organisms were noticed on the media the next day, the material was inseminated directly on to the special culture media (for growing T. B.) and also inoculated subcutaneously and intraperitoneally into guinea-pigs.

In case of tissues obtained at autopsy and in case of contaminated materials in general, preliminary digestion had to be resorted to with either alkalis or acids. Knowing that any caustic agent, which is intended for eliminating contaminations, will destroy the viable forms of tubercle bacilli according to the resistance of the coat of the bacilli, the strength of the reagent and the length of exposure, we carried out a series of preliminary experiments with different dilutions of both acids and alkalis and digesting the materials for varying lengths of time. In the case of alkalis, we tried a 7.5 per cent solution of antiformin and a 4 per cent solution of NaOH. Twenty to thirty minutes' exposure to antiformin and half an hour's digestion with 4 per cent NaOH were found to be suitable for successful cultures. For an acid digestion 20 per cent, 10 per cent, and 6 per cent solutions of  $\text{H}_2\text{SO}_4$  were employed. Half an hour's digestion with 6 per cent  $\text{H}_2\text{SO}_4$  was found to destroy contaminating micro-organisms, while yielding a positive growth of tubercle bacilli in a majority of cases. It must be mentioned that these dilutions gave the maximum number of positive cultures, but there are some strains and young forms which are very susceptible to chemical treatment. In one case, we obtained a positive culture after only 3 minutes' digestion with 4 per cent caustic soda, while longer exposures proved sterile. The same technique of culture was used after treatment with the different solutions.

In order to find out which of the two methods of digestion, alkali or acid, yielded better results, a comparative experiment was undertaken with positive sputum. After half an hour's digestion with 4 per cent NaOH and 6 per cent  $\text{H}_2\text{SO}_4$ , they were cultured on Dorset's and Hohn's media. The following table shows the proportion of successful cultures and contamination by each method :—

TABLE I.

Digestive employed.	6 per cent $\text{H}_2\text{SO}_4$ .	4 per cent NaOH.
Tubercle bacilli recovered on culture in per cent.	75.0	62.5
Tubes contaminated in per cent	51.5	63.6
Average time required for demonstrable growth.	22 days.	32.4 days.

It appears that sodium hydroxide, in the dilution and the duration of exposure employed, has been more detrimental to the tubercle bacilli than  $\text{H}_2\text{SO}_4$ . Some workers (e.g., Sweaney and Evanoff, *loc. cit.*) think that, although NaOH is more destructive for tubercle bacilli, it has greater concentrating power than  $\text{H}_2\text{SO}_4$ . Others (e.g., Loewenstein, Sumoyoshi, Hohn, Corper and Uyei, *loc. cit.*), on the other hand, think that, with the acid method, the growth appears a few days earlier and

a larger percentage of successful cultures is obtained. Our results support the latter view. Since these observations were made, we have employed the acid digestion method only in our subsequent experiments.

*Media employed.*—After ascertaining the method of digestion, we proceeded to choose the most sensitive medium for culture. With this purpose, a sample of sputum with moderate numbers of tubercle bacilli was digested and inseminated into Hohn's, Corper and Uyei's, Petraghini's, Woolley and Petrik's, and Lubenau's modification of Dorset's medium. Of these, Hohn's and Lubenau-Dorset's media do not contain any dye, Woolley and Petrik's (*loc. cit.*) and Corper and Uyei's contain crystal violet and Loewenstein's and Petraghini's (*loc. cit.*) contain malachite green.

The sputum was mixed with an equal quantity of  $\text{H}_2\text{SO}_4$  and thoroughly shaken. It was next placed in the incubator at  $37^\circ\text{C}$ . for half an hour and then diluted with 10 times the volume of normal saline. After shaking thoroughly, the whole was centrifugalized at high speed for 15 minutes. The supernatant fluid was then decanted off, leaving a thin layer above the sediment. This was then thoroughly mixed up and three loopfuls of the emulsion were spread and well rubbed on the surface of two tubes of each kind of media. The culture tubes were then placed in an incubator at  $37^\circ\text{C}$ . and were examined after 24 hours to see if any contamination had taken place. Thereafter they were examined at weekly intervals with reference to the rapidity, amount and character of growth as also to any contaminations that might have taken place. It was also noticed that the media, whose surfaces were rubbed with sterile spatula after a few days of incubation, showed visible growth more easily. As soon as a visible growth was detected, a microscopic examination of smears from surface scraping was done. In case of the digestion of pathological tissues, they were thoroughly ground with pestle and mortar before being mixed with  $\text{H}_2\text{SO}_4$ . The subsequent treatment was identical with that of the sputum. It was observed that the growth was generally facilitated by rubbing the surface of the inoculated media, with a sterile spatula, a few days after insemination.

Table II illustrates comparative data obtained by culturing sputum on different media :—

TABLE II.  
*From smear-positive sputum.*

Name of medium.	Hohn's.	Corper and Uyei.	Petraghini.	Woolley and Petrik.	Lubenau-Dorset.
Per cent recovered by culture ..	40	54.1	83.3	80	40
Per cent tubes contaminated ..	32	12.5	Nil	Nil	40

It will be seen that Petraghini's and Woolley and Petrik's media gave decidedly better results than the other three media. The following results (Table III), obtained by culturing other pathological materials, such as pus and pleural fluid

TABLE III.  
(From pathological materials.)

Materials examined.	Number examined.	Per cent positive in smear.	CORPER AND UYEL.		PETRAONINI.		WOOLLEY AND PETRIK.		LUDENAU'S MODIFIED FORSET.	
			Per cent recovered by culture.	Per cent contaminated.	Per cent recovered by culture.	Per cent contaminated.	Per cent recovered by culture.	Per cent contaminated.	Per cent recovered by culture.	Per cent contaminated.
Pus and pleural fluid.	131	16.7	9.3	9.3	19.0	5.7	16.4	10.6	13.9	18.8
Post-mortem tissues.	185	19.4	5.5	15.5	28.6	12.5	24.2	11.8	19.6	28.3
Both combined	316	18.3	6.7	13.5	24.4	9.5	21.0	11.3	16.8	23.6



and post-mortem tissues, will further prove the superiority of the Petraghini's and Woolley and Petrik's media. The autopsy materials were derived from 14 cases who died of pulmonary tuberculosis, 15 from injury and accidents and the remainder from non-tuberculous diseases. Of the materials in Table III, all the post-mortem tissues and 24 out of the 131 samples of pus and pleural fluid underwent treatment with chemical digestion before culture. Of the 57 positive cultures obtained, 28 strains were recovered from the tissues of cases who died of pulmonary tuberculosis.

It will appear from both these experiments that the Petraghini medium is perhaps the most sensitive of the media studied by us for the growth of the tubercle bacillus from pathological materials. It will be further noticed that the culture method gives more information about the presence of T. B. in pathological materials than in smears obtained by concentration method.

*Inhibiting substance.*—The purpose of the addition of a dye to the medium is to inhibit the growth of such contaminating micro-organisms as are not destroyed by the previous treatment with digestives and also to facilitate the early detection of the growth of tubercle bacilli as a result of the contrast between the cream colour of the colonies and the coloured background of the medium. Corper and Uyei (*loc. cit.*) tested the action of crystal violet or related dyes, sold under the name of gentian violet, on the growth of tubercle bacilli. They found that at a concentration of 0.01 and 0.002 per cent growth occurred equally well to that in control (without dye) and that crystal violet was slightly more bacteriostatic than gentian violet. In our experiments there was slightly less contamination and slightly more percentage recovery of tubercle bacilli with 0.06 per cent malachite green (Petraghini) than with 0.003 per cent crystal violet (Woolley and Petrik, *loc. cit.*).

#### CULTURE *versus* ANIMAL INOCULATION IN ISOLATING TUBERCLE BACILLI.

*Technique of culture.*—In case of *sterile* materials like pus and pleural fluid, the bacilli have been isolated by direct culture of the material on Petraghini and Woolley and Petrik medium in about 23 per cent of cases. Two drops of the material are inseminated in two tubes of each type of medium. Concentration by digestion and centrifugalization kills, as has been said, a certain proportion of the bacilli. In case of *contaminated* materials, they were treated with chemicals as described anteriorly. Three loopfuls (2 mm. diameter) of the centrifugalized sediment were inseminated into two tubes of the above two types of media, incubated and examined from time to time as described above.

*Guinea-pig inoculation.*—In case of *sterile* materials, like pus or pleural fluid, about one c.c. (or 8 to 10 times the amount used for culture) was inoculated subcutaneously into the right inguinal region of two guinea-pigs and intraperitoneally into a third. In case of *contaminated* materials, 3 to 4 times the amount utilized for culture were introduced subcutaneously into the right inguinal region of two guinea-pigs.

The inoculated guinea-pigs were weighed and examined every week. If a positive culture was already obtained from a particular material or if the animals showed enlarged glands near the site of inoculation or progressive emaciation, they were sacrificed; otherwise every animal was kept under observation for 100 days

before being sacrificed. On autopsy, smears were obtained from the site of inoculation, the contents of the inguinal, pelvic, mesenteric and tracheo-bronchial glands and from the spleen, liver and lungs, in case if any suspicious spots were detected in these organs. The smears were examined by Ziehl-Neelsen stain and carefully examined under the microscope. In case the smear proved positive, a culture was made from the affected tissues.

*Results of culture and animal inoculation.*—The materials included in Table IV were utilized for both culture and guinea-pig inoculation. Of the 288 materials examined, 44 showed tubercle bacilli in smears under the microscope, 14 of which showed tubercle bacilli only after one of the concentration or homogenization methods was employed. The findings are noted in Table IV :—

TABLE IV.

Nature of materials examined.	Number examined.	NUMBER SHOWING T. B. IN SMEAR.		Totals.
		Untreated.	After homogenization.	
Pus and pleural fluid ..	111	16	2	18
Skin .. ..	2	..	..	..
Urine .. ..	5	..	..	..
Stool .. ..	3	1	..	1
Stomach washing ..	1	..	..	..
Sputum .. ..	10	1	1	2
Autopsy tissues ..	156	12	11	23
TOTALS ..	288	30	14	44

Out of 44 smears positive mentioned in the above table, successful cultures were obtained from 32 of them, while in 26 cases inoculated guinea-pigs showed tuberculous lesions.

Among the total number of materials examined, tubercle bacilli could be demonstrated in 102 materials either by culture or by animal inoculation or by both. Of these, 40 were positive by culture alone, 27 by animal inoculation alone and 35 by both the methods. Thus if we take 35 as the common factor, we arrive at the total figures given in Table V. That is, cultures gave us positive information in 75 cases and animal inoculation in 62 cases, or a superiority of 12·8 per cent more information with cultures (taking 102 positive materials into account). Out of 156

autopsy tissues examined, 24 showed giant cells in section, of which 16 showed tubercle bacilli in smears. From these, 17 strains were recovered by culture alone, while 10 showed tuberculous lesions by animal inoculation. Table V will illustrate the position regarding the sensitiveness of the two methods:—

TABLE V.

Nature of materials examined.	Number examined.	Per cent positive in smears.	CULTURE.		ANIMAL INOCULATION.		Number showing giant cells in sections.
			Number positive.	Per cent positive.	Number positive.	Per cent positive.	
Pus and pleural fluid.	111	16.2	32	28.8	30	27.0	..
Skin ..	2	..	..	..	1	50.0	1
Urine ..	5	..	1	20.0	1	20.0	..
Stool ..	3	33.3	1	33.3	1	33.3	..
Stomach washing	1	..	1	100.0	..	..	..
Sputum ..	10	20.0	1	10.0	3	30.0	..
Autopsy tissues	156	14.7	39	25.0	26	16.6	24
TOTALS ..	288	15.2	75	26.0	62	21.5	..

The earliest period at which a culture became positive was 5 days, while the earliest positive finding (demonstration of tubercle bacilli) was obtained from the inguinal gland of an inoculated guinea-pig on the 12th day. The maximum period for a positive finding was the same (100 days) in both. The average period for positive finding was found to be 33.8 days in the case of culture and 48 days in the case of animal inoculation.

## DISCUSSION.

It will be noticed that most of our material was derived from non-pulmonary sources and from contaminated material from post-mortem tissues. Although lately the question of culture of tubercle bacilli as a method for diagnosis has attracted attention, yet enough work has not been done with materials from non-pulmonary and autopsy sources. If the material is sterile, the question of a digestive for the destruction of secondary organisms does not arise. A digestive has to be used in the case of septic materials, but, in our efforts to kill or inhibit the growth of secondary organisms in this case, a certain proportion of tubercle bacilli is killed and 'denatured'. In comparing the results of culture and animal inoculation, it should be remembered that a small amount of material is required for culture, while a much larger amount is introduced into the guinea-pig.

For a successful culture, the medium should be carefully selected to produce a sort of selective environment, the material highly concentrated and evenly planted. In spite of all precautions, a certain amount of contamination, due to various causes, is bound to occur. It will be noticed from our findings that the employment of two different kinds of media gives a higher percentage of successful cultures than only one kind of medium. The number of tubes seeded is also important. As has already been stated, the guinea-pig receives 8 to 10 times the material seeded for culture. Hence, for comparable results 8 to 10 tubes (or 4 to 5 if two different kinds of media are employed) should be inoculated, but we used half this number in our experiments. Even, in spite of this, the culture method has given higher results in about one-third of cases, particularly in case of autopsy materials. But, at the same time, animal inoculation has given positive results in a fair number of cases where culture had proved negative. In 46 cases where tubercle bacilli were not found on direct smear examination, the culture gave positive results. Hence, as a diagnostic method it seems to be superior to other methods employed in the hospital.

From the evidence adduced by us it seems that although the culture method is more sensitive than guinea-pig inoculation, it cannot be dispensed with altogether, as a combination of both the methods gives us a higher percentage of positive findings. In addition to the cheapness of cost and ease of technique, the culture method saves considerable time and allows an opportunity to proceed at once with the typing (bovine, human or avian) tests. Of all the culture media we have worked with, the Petraghini medium seems to be superior to others as regards sensitiveness for the growth of tubercle bacilli.

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## A STUDY OF NERVE LEPROSY.

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THIS paper records the results of the study of eighty-one cases of leprosy. The history and clinical findings were carefully ascertained and a pathological and bacteriological study of the lesions was made in biopsy material.

The object of this study has been to find out the path of infection in nerve leprosy, the nature of the pathological process inside the nerves, and the way in which this pathological process brings about various clinical phenomena.

### GENERAL CLINICAL DESCRIPTION OF CASES.

Of 81 cases, 71 were classified under nerve leprosy. Of these, 51 showed only single lesions (N-1) and 21 multiple lesions (N-2). Of the remaining 7, 2 were slight cutaneous (C-1) and 5 were more advanced cutaneous (C-2 or C-3).

We have selected for the most part 'initial' cases, i.e., those in which the disease had not advanced far, though in some the duration was considerable, and it will be noticed that in 53 of them there was only a single observed lesion. In the majority of these there was, in addition to the affected nerve, and connected with it, a definite visible skin lesion with a more or less circumscribed margin. This lesion showed such signs as anæsthesia when touched with a feather, absence of pain when pricked with a pin, keratosis, hypopigmentation, depilation, anhydrosis, erythema, induration and visible or palpable thickening. In some cases erythema,

induration and thickening were uniformly marked throughout the lesion, and in others they were confined to the margin, the centre being comparatively thin, flat and pale. Outside the border of such lesions there was frequently an area of skin showing no visible change, but with anæsthesia to light touch and pain. In a few cases no visible skin lesion was present, but the normal-looking area of skin supplied by an affected sensory nerve branch was anæsthetic.

The length of nerve with palpable involvement (thickening and tenderness) varied in different cases. In some only a small branch was thickened for a distance of a few inches; in others more than one sensory branch supplying the affected skin area was thickened; while in others again one or more mixed nerve trunks, such as the ulnar, showed thickening and tenderness on pressure.

In some cases the thickening of the nerve was uniform, while in others the course of the nerve branch was dotted with a series of small nodules, the inter-nodular nerve lengths being palpable but considerably thinner. This was particularly noticeable in branches of the lateral cutaneous nerve of the forearm. These nodules, when incised or sectioned, were found to contain caseous matter and sometimes white pus.

The small nerve branches, when thickened, seldom gave rise to pain, unless they happened to be situated over a bone, as at the elbow or knee, against which the nerve might be compressed by any hard object striking against it.

When larger mixed nerve trunks were affected, pain was a much more common phenomenon. This was especially frequent in the ulnar nerve; and here in addition to pain in the thickened nerve there was frequently pain in the corresponding hand, this being accompanied by anæsthesia and wasting of the small muscles of the hand and occasionally, through disuse of the hand, of muscles of the forearm. In most of these nerve-trunk cases there was a large or small visible anæsthetic lesion of the skin in the distribution of the ulnar nerve, but the area of anæsthesia extended far beyond the visible lesion.

In most cases with ulnar involvement the nerve showed palpable thickening only in its superficial course, that is below the wrist and above the elbow. But in some cases abnormal tingling could be elicited by striking the forearm over the nerve in the intermediate space, showing that this portion of the nerve was also affected to a certain extent.

#### BACTERIOLOGICAL FINDINGS.

The 71 neural cases were classified as such partly in virtue of the fact that we failed to find bacilli in the skin but in 51 of them (35 N-1 and 16 N-2) bacilli were found in one or more nerves. Bacilli were found in varying numbers both in the skin and nerves of all the cutaneous cases.

Caseation or abscess formation was found in the nerves of 19 (13 N-1 and 6 N-2) of the neural cases and in 2 of the cutaneous (C-2) cases (in one of these as the result of intravenous mercurochrome injections), and bacilli were seen in connection with all of these with the exception of one.

The following Table gives a list of the nerves examined and the number under each nerve found positive and negative :—

TABLE.

Name of nerve.	Number positive.	Number negative.	Totals.
Ulnar .. ..	20	5	25
Radial .. ..	8	4	12
Medial anti-brachial ..	10	5	15
Femoral cutaneous ..	0	1	1
Great auricular ..	2	3	5
Sural .. ..	2	1	3
Peroneal .. ..	9	2	11
Supra-clavicular ..	1	1	2
Infra-patellar ..	2	0	2
Supra-orbital ..	1	0	1
Saphenous ..	1	0	1
Dorsal anti-brachial ..	2	0	2
TOTALS ..	58	22	80

In all the nerves examined, whether they showed acid-fast bacilli or not, considerable cellular granuloma was present.

#### DESCRIPTION OF SELECTED CASES.

To save space we have chosen 37 typical cases out of the total 81. We have written short descriptions of them and have illustrated them by means of 27 photographs and 24 sketches. For easy reference the figures which appear on the illustrations correspond with the numbers of the cases.

#### *Cases illustrated in Plate I.*

*Case 12a*, abscess of ulnar nerve just proximal to elbow. The capsule of the abscess is formed of the thickened and stretched epineurium. In *Case 12b* the abscess is shown opened. In the base of the cavity is the thickened nerve. The needle indicates the opening through which white pus has emerged from the interior of the nerve and distended the abscess cavity. Above and below the abscess is shown the thickened ulnar nerve and a thickened branch. A few acid-fast bacilli



induration and thickening were uniformly marked throughout the lesion, and in others they were confined to the margin, the centre being comparatively thin, flat and pale. Outside the border of such lesions there was frequently an area of skin showing no visible change, but with anæsthesia to light touch and pain. In a few cases no visible skin lesion was present, but the normal-looking area of skin supplied by an affected sensory nerve branch was anæsthetic.

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#### *Cases illustrated in Plate IV.*

*Case 5a* shows a section (low power) of a small piece of nerve tissue removed from the thickened distal branch of the superficial peroneal nerve in a C-1 N-1 case. *Cases 5b* and *5c* show increasing magnifications of parts of the same section. In the latter, large numbers of bacilli are visible, but comparatively little cellular response. Smears from the skin showed a few acid-fast bacilli in a raised, erythematous and anæsthetic macule. The section was stained by Ziehl-Neelsen and hæmatoxylin. There was only one small skin lesion found on the dorsum of the left foot.

*Case 7a* is from a section of a nerve in a C-3 case. It is stained by Ziehl-Neelsen and a modification of Bielchowsky's method, so as to show both bacilli and nerve fibres. Note the masses of bacilli without destruction of nerve fibres. *Case 7b* is a higher magnification of part of the same section. Note absence of infection of the nerve sheath in the upper part of the photo.

*Case X* does not belong to this series of cases. It is a photomicrograph taken from a leprous nerve section stained by modified Bielchowsky's method. The nerve is cut obliquely. In the centre is a granular mass which has destroyed the nerve fibres. At the margin the nerve fibres are seen stained black. The appearance, on moving the field of the microscope and focusing different planes, was that of a stick of asparagus surrounded by horsehairs.

#### *Cases illustrated in Plate V.*

*Case 3* shows massive infection in a section of a thickened branch of the right sural nerve. This was a N-1 case with an anæsthetic patch on the foot. There was a granulomatous reaction to the bacilli, but no caseation. The only other lesion was a small depigmented patch on the back. Smears from the skin were bacteriologically negative.

*Case 4a* shows section of branch of dorsal anti-brachial cutaneous nerve. Note caseation of centre of the branch and the persisting nerve fibres at the margin; no bacilli were found in this part. *Case 4b* shows a teased nerve fibre taken from another part of the nerve with acid-fast bacilli but no cellular reaction. The corresponding skin lesion is shown in Plate VI, Case 4.

*Case 19* shows teased nerve fibres taken from slightly thickened supra-orbital nerve. The corresponding skin lesion was raised and erythematous, but not anæsthetic to light touch. Cellular response was slight.

*Case 78* shows section of capsule of peroneal nerve in a C-2 N-2 case. Note the intracellular position of the bacilli.

*Cases illustrated in Plate VI.*

*Case 1* shows front and back of left upper limb with anæsthetic lesion of hand, raised and erythematous except in the centre which was flat and hypopigmented; abscess of ulnar nerve, after opening of which pain and tingling were less, but anæsthesia as before; thickening of the branch of the lateral cutaneous nerve with two globular swellings, which on removal and section showed caseation without liquefaction. No bacilli were found in skin smears, or in nerve sheath or fibres, but several single acid-fast bacilli were found in the mass of caseous material.

*Case 4*, scar due to application of corrosive to leprous lesion  $1\frac{1}{2}$  years previously; margin spreading at two points where red and raised; thickened branch of lateral cutaneous nerve which on section showed centrally a necrosed area with new fibrous tissue formation. Acid-fast bacilli were found in section in areas bordering on the necrosis and also between teased nerve fibres taken from branch (*see* Plate V, Cases 4a and 4b).

*Case 12* shows anæsthetic and hypopigmented patch of hand and abscess of ulnar nerve; inset shows form of abscess opening through which pus emerged, caseous material coating inner side of capsule, branches of medial cutaneous nerve of arm spread out over capsule (*see* Plate I, Cases 12a and 12b).

*Case 13* shows a raised, erythematous and anæsthetic patch at the back of the left wrist with thickening of the terminal distal branches of the dorsal cutaneous nerve of the forearm, and an abscess of this nerve above the elbow. A teased preparation from the branch in the neighbourhood of the skin lesion showed no bacilli; a smear from the pus of the abscess showed six bacilli after examining 100 fields. The ulnar nerve was thickened above the elbow; after removal of the nerve sheath tingling of the fingers diminished, but anæsthesia remained the same. No other lesion was found in the body.

*Case 15* shows a small patch, raised, erythematous and anæsthetic at the back of the elbow, and thickening of the supplying branch of the medial cutaneous nerve of the forearm. No bacilli were found in skin or nerve, but the latter was highly granulomatous and showed abundant giant cells.

*Case 16*, raised, erythematous and anæsthetic lesion of right thumb, with swelling of the supplying nerve branch. On removing and teasing a few nerve fibres from the latter, two acid-fast bacilli were found lying on a fibre. The skin showed no bacilli. This was the only lesion.

*Case 17*, an N-2 case with multiple, raised, erythematous, anæsthetic, lesions scattered over the body. Smears and sections taken from the thickened sural nerve did not show any acid-fast bacilli.

*Case 18* showed a small red, raised and anæsthetic patch just above the back of the middle finger, but anæsthesia extended as far as the wrist. A branch of the radial and two branches of the ulnar nerve showed thickening. On examining the latter, some parts were found granulomatous and others not. In the non-granulomatous parts of the nerve bacilli were found singly and in bunches. Smears from the skin showed no bacilli.

*Case 21*, an N-2 case with multiple anæsthetic patches scattered over the body and palpable thickening of no fewer than 10 nerves. A patch on the back of the left hand was found with thickening of the supplying branches of the ulnar nerve.

Inset a caseous swelling is shown at the bifurcation of a branch of this nerve. A smear from the caseous material showed six bacilli in 250 fields. A teased portion of the nerve showed absence of granuloma, but large numbers of acid-fast bacilli lying between the nerve fibres. After the excision of the swelling and adjacent nerve there was a certain degree of restoration of sensation to the affected skin. Smears from the skin were negative.

Case 22 shows a lesion of the left leg, the only one found in the body, and thickening of an infra-patellar nerve branch. Smears from the skin were negative, but sections of the nerve showed widely scattered masses of bacilli. The nerve sheath showed no bacilli.

Case 27, an N-2 case with numerous small patches, smears from which were negative. The diagram shows a patch on the lateral aspect of the right knee with thickened nerve branches, one of which has a globular swelling (inset). Three bunches of bacilli were found in the caseous material, but none in the cellular part of the swelling.

Case 29 shows a small raised and anæsthetic patch on the back of the right ring finger, thickening of the supplying branch of the ulnar nerve and also of this nerve above the elbow. The skin smear was negative; two bacilli were found on some fibres removed from the nerve branch.

Case 36 shows a leprous lesion of the skin of the back of the right hand; the front of the thumb was also affected; the centre was flat and hypopigmented, but the margin raised and erythematous; the whole area was anæsthetic to light touch. The ulnar nerve above the elbow was thickened and tender. No bacilli were found in the skin or in the scrapings of the nerve capsule or fibres. Removal of the nerve sheath resulted in great reduction of pain both above the elbow and in the hand, but not of anæsthesia.

Case 37 shows a hypopigmented and anæsthetic area on the back of the right hand with thickening and abscess formation of the ulnar nerve above the elbow. The abscess was opened and the thickened part of the nerve decapsulated. No bacilli were found in the skin, nerve capsule or fibres, but the pus from the abscess contained several large bunches of bacilli and many single bacilli.

Case 39 shows a large lesion of the medial side of right arm with hypopigmentation and anæsthesia, thickening of the medial cutaneous nerve of the forearm and its branches. There was also marked thickening of the ulnar nerve above the elbow. Smears from the skin lesion showed no bacilli, but examination of the medial cutaneous nerve showed a large necrosed area with one bunch of acid-fast bacilli outside the necrosed area but no bacilli in sheath. Duration seven years; gradual extension of lesion at times of recurrent fever attacks.

Case 46 shows an anæsthetic area on the back of the right hand with parts of the distal margin raised and erythematous; both radial and ulnar nerves were markedly thickened. Smears from the skin failed to show bacilli. Fibres of the thickened radial nerve were removed, but failed to show bacilli, the nerve showed a highly granulomatous state (see also Plate II, Case 46).

Case 47 shows a small, flat, hypopigmented anæsthetic patch on the right hand. The thickened branch of the medial anti-brachial nerve supplying the part was excised resulting in almost entire restoration of sensation to the part. A smear

taken from the proximal part of the excised nerve showed 4 or 5 bacilli and a smear from the distal part 10 or 12 bacilli. A smear from the skin was negative.

*Case 48* shows a patch on the left forearm, raised, erythematous and anæsthetic; also thickening of the supplying branches of the medial and dorsal anti-brachial nerves. The former shows pea-like thickenings and this portion was excised and sectioned. It showed densely granulomatous nerve bundles with small giant cells. In one bundle there was necrosis with fragmentation of nuclei and formation of fine, reticular fibrous tissue. Excision of the nerve branch produced no change in sensation.

*Case 50* shows a large anæsthetic lesion of the forearm, flat and hypopigmented in the centre, raised and erythematous at the margin, with marked thickening, pain and tingling of the ulnar nerve. The nerve sheath was excised resulting in reduction of pain and thickening and giving a lighter feeling in the hand and partial restoration of sensation at the elbow. The thickened epineurium with attached muscular septum was sectioned, but showed no acid-fast bacilli. A few nerve fibres were removed and teased out on a slide; they showed two bacilli in 100 fields. Smears of the skin were negative for bacilli.

*Case 54* shows a lesion of the entire right forearm marked by slight hypopigmentation and diminished pain sensation, but with anæsthesia to light touch only behind the elbow. The hand was apparently quite normal. Two branches of the medial anti-brachial nerve and a branch of the radial were thickened. One thickened branch of the former nerve was removed for examination. Sections showed cell-infiltration in foci. There were no bacilli found in the highly granulomatous foci or in the sheath, but a few were found in the slightly granulomatous foci and many bacilli were found lying between the nerve fibres in the otherwise normal-looking areas. In the latter the bacilli were chiefly single, but a few small bunches were found. Smears from the skin failed to show bacilli. The above were the only lesions found in the body.

*Case 59*, patient of 28 years, who gave a history of having contracted syphilis seven years previously, showed a small anæsthetic area on the back of the left hand and very slight thickening of the supplying branch of the radial nerve. There were also two minute suspicious patches, one on the back of the right hand, and one on the right ankle. The thickened nerve branch was excised for examination, followed by partial restoration of sensation in the anæsthetic area. Sections of the nerve showed one distinct acid-fast bacillus lying on a nerve fibre. Skin smears showed no bacilli.

*Case 68* shows a raised, red and anæsthetic patch above the back of the right wrist with two small subsidiary lesions. The irregularly thickened medial anti-brachial nerve branch was dissected out and is represented in the inset. There was a similar lesion with thickened nerve branch on the dorsum of the left foot and a lesion on the left cheek. These lesions were not stained for bacilli.

*Case 81* shows a flat hypopigmented, anæsthetic lesion of the back of the right forearm, with a thickened branch of the dorsal anti-brachial cutaneous nerve. At the point where this branch divides was an abscess. The abscess was evacuated and showed bunches of acid-fast bacilli. The smaller terminal branch was excised and sectioned, but no bacilli were found. Smears from the skin were negative for bacilli. No other lesion was found.



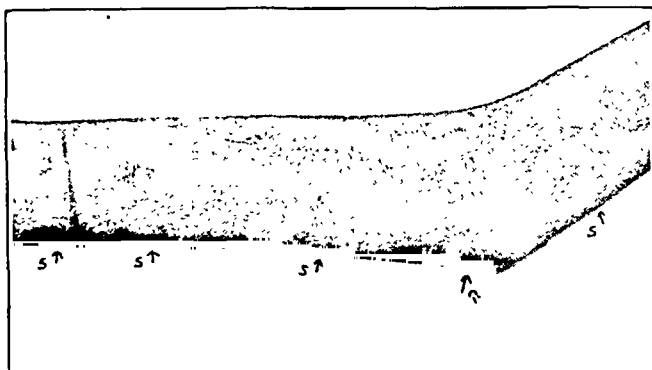
Case 12a.



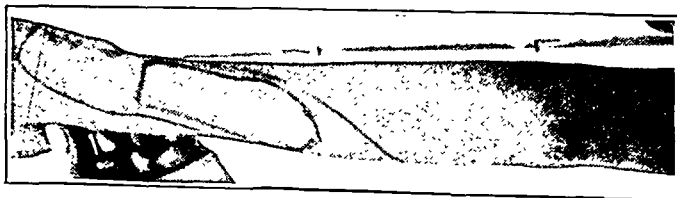
Case 12b.



Case 9.



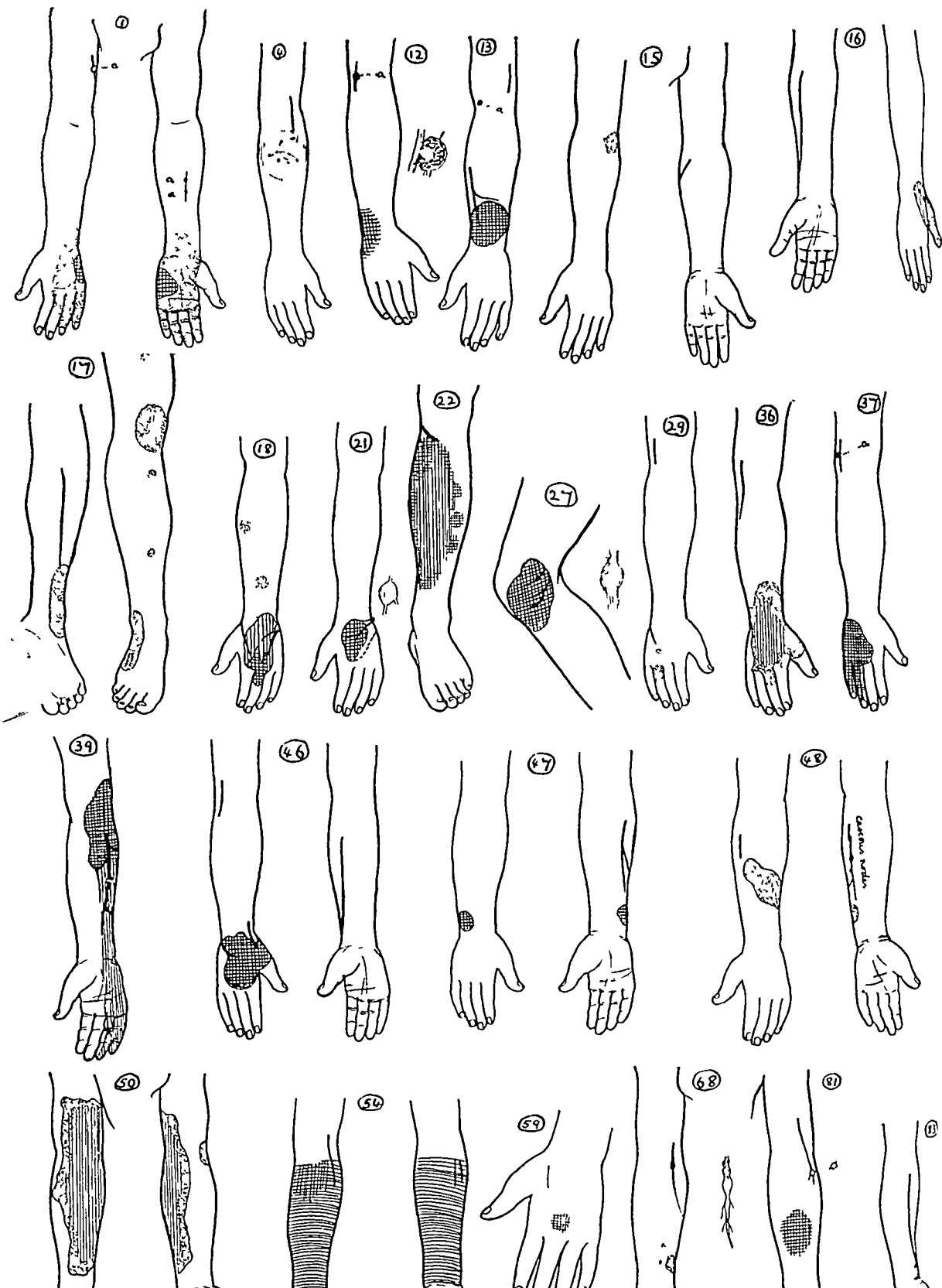
Case 8.



Case 63.

# PLATE VI.

*Note.*—Case numbers are indicated by encircled figures.



Case 83. See also Plate II. Cases 83a and 83b, under which this raised, red, anæsthetic lesion with the thickened branch of the radial nerve is described.

### DISCUSSION.

#### *Entry of bacilli and their passage up nerve bundles.*

There is considerable evidence to show that *M. lepræ* enter the nerve bundles by way of the skin, passing up sensory nerve branches from the subpapillary and subcutaneous nerve plexus. It is frequently seen that the only nerves affected are those which supply sensory branches to a skin lesion, this being the only lesion present in the body. This is well illustrated in Plate VI, Cases 1, 13, 18, 48 and 54. It is obvious in these cases that infection could not have entered the nerves direct through their blood supply or otherwise than by passing up from the skin.

Infection may spread up a nerve from the skin causing little or no palpable or visible change in the nerve, until it reaches a point of obstruction at which it causes thickening and tenderness (Plate VI, Cases 1, 13, 36, 37, 46 and 81).

Often, however, there may be thickening of a local nerve branch, with little or no apparent skin lesion connected with it, as is seen in Plate VI, Cases 15 and 83, and Plate II, Cases 10 and 83. In other cases, a large mixed nerve such as the ulnar above the elbow, is found thickened, without any lesion of the skin area which it supplies but, as is shown later, there is reason to believe that in at least the majority of such cases entry of bacilli has been through the nerve endings from the skin.

When we section the corium, we find nerves and blood vessels running in close proximity to each other in the subpapillary and subcutaneous plexuses, and round the hair follicles and coil glands. This same proximity is found in the small neurovascular bundles in the subcutaneous tissue. It is in close connection with nerves and vessels in these plexuses and round these epithelial extensions that *M. lepræ* are found in sections of recently infected skin. The organisms lie extra- and intracellularly, and excite varying degrees of cellular response, especially from the endothelial cells of the capillaries.

It appears that the bundles of peripheral sensory and mixed nerves form a specially favourable medium for the multiplication of *M. lepræ*, considerably more favourable than either the skin or the connective tissue sheaths of these bundles (perineurium and epineurium). This is shown in several of our cases, and especially in those illustrated in Plate VI, Cases 4, 16, 22, 39, 50 and 54, Case 4 also being illustrated in Plate V. In these cases there were large and conspicuous leprosy lesions of the skin. No bacilli were found in smears made from the skin or from the perineurium, but bacilli were present (sometimes in large masses) lying between the nerve fibres.

It should be noted here that while the skin was examined, except in a few cases, only by means of smears of material scraped with a sharp knife from the corium, the nerves were examined by scraping and teasing the nerve fibres, and in some cases by sectioning. The more thorough examination of the nerves does not, however, at all account for the greater number of bacilli found in the nerves, as a careful study of the case records will show.



*Concentration of bacilli in nerves at certain points.*

Under favourable circumstances *M. lepræ* gradually pass up the nerve bundles from the skin, and are found lying between the medullary sheaths of the nerve fibres. They do not penetrate the medullary sheaths. They are probably propelled up the course of the bundles by the lymph flow, and multiply as they pass up. They tend to accumulate at certain points where they meet with obstruction. This obstruction is caused by bending or branching of the nerve, by its constriction under fibrous bands, or by the nerve passing superficially over bony surfaces where it is liable to injury. The results of such accumulations of *M. lepræ* are mentioned later.

One of the commonest sites for accumulation of *M. lepræ* is the stretch of the ulnar nerve between the elbow and the middle of the arm. At the former point it passes between the medial epicondyle of the humerus and the olecranon, and is bound down by a more or less dense and narrow arch of deep fascia; at the latter point it passes behind the intermuscular septum. At both of these points, and especially at the former, the nerve tends to be constricted; and the intermediate stretch of nerve is liable to injury. The constant bending of the nerve also favours the obstruction of the passage of bacilli up the nerve. Hence this is the commonest site of nerve lesions, as is shown by reference to Plate VI, Cases 1, 12, 29, 36, 37 and 46. Another reason for the frequency of affection of the ulnar nerve is the large skin area with which it is connected, and the peculiar liability to injury of that skin surface. Thus the ulnar nerve has through its cutaneous terminals a peculiarly wide and favourable catchment area for *M. lepræ*.

Similarly, the superficial peroneal, great auricular and supra-orbital nerves are liable to injury, and *M. lepræ* in passing up them are subject to obstruction; hence these nerves, next to the ulnar, are among those most commonly affected.

*Cellular response to bacilli.*

We have mentioned above that the bundles of peripheral nerves form a more favourable medium for the multiplication of *M. lepræ* than do the skin and the connective tissue sheaths of these bundles. Hence *M. lepræ* may be said to be 'neurotropic', applying this term in a special sense, quite different from that generally applied to certain viruses. What this neurotropism is due to it is difficult to say. Attempts have been made to culture *M. lepræ* on nerve fibres, but no better success has yet been attained with this than with other media.

Our present study, however, tends to show that one of the important factors which permit the passage of *M. lepræ* up the nerves and their multiplication between the nerve fibres is the partial or complete absence of cellular response to the organisms as compared with the much greater response in the skin and in the neural connective tissue. This is well illustrated in the cases referred to at the end of the Section on the entry of bacilli and their passage up nerve bundles but especial reference may be made to Case 22 (Plate VI) in which, in spite of an intense degree of cellular granuloma in the skin, along with negative bacteriological findings in both skin and nerve sheath, there were masses of bacilli found in the small supplying nerve branch. Cases 5 and 7 (Plate IV) and Case 82 (Plate III) also show the intense bacillary infection that may be present in the nerve bundles with comparatively

little cellular response. As is mentioned in Case 54 (see Plate VI) there were no bacilli found in the skin or nerve sheath, there were no bacilli found in the highly granulomatous foci in the nerve, a few bacilli were found in the slightly granulomatous nerve areas, but many bacilli were seen lying between the nerve fibres in the otherwise normal-looking areas.

### *Source of cellular response in nerve bundles.*

In the skin the principal cells responding to the presence of *M. lepræ* are the endothelial cells of the capillaries. As the bacilli in the skin lie in close connection with the capillaries and their cells, the latter respond easily to their contact. The same holds good in the vascular connective tissue surrounding the nerve bundles, as is shown in Plate V, Case 78, and Plate II, Case 83; in the latter the cellular proliferation belongs to the perineurium, in which no bacilli are seen; the bacilli are lying on a few adherent nerve fibres.

In the nerve bundles, on the other hand, the vascular capillaries lie centrally, and the bacilli are more or less isolated from the endothelial cells of those capillaries by the medullated fibres. Although the bacilli are apparently more or less uniformly distributed throughout the bundle to begin with, it is at the centre that response first takes place causing granulomatous accumulation and bacillary ingestion and phagocytosis. This spreads outward displacing, blocking and destroying the central nerve fibres till the core of the bundle becomes a granulomatous mass with nerve fibres remaining only as an outside covering. The appearance of such a nerve bundle on longitudinal section, when stained with a special nerve stain, may be compared to a stock of asparagus covered with black horsehairs (see Plate IV, Case X). Such sections will often show an absence of bacilli at the centre, where they have been destroyed by phagocytosis, and their presence at the periphery to which the phagocytic cells have not yet reached. This is well shown in Plate III, illustrating Case 82. In Case 7, illustrated in Plate IV, practically no cellular response has taken place, and massive infection is seen between the nerve fibres; while the perineurium surrounding the bundle is practically free from bacilli. Also in Case 5, illustrated in the same plate, an intermediate stage is shown; a mild degree of cellular reaction has taken place, some bacilli having been ingested by the cells while others lie extracellularly.

### *Results of varying degrees of cellular response in the skin.*

We have stated above that cellular response to lepra bacilli is, in the same subject, stronger in the skin and in the neural connective tissue than in the nerve bundles. The degree of cell response also varies in individuals, being stronger in some and weaker in others. It also is liable to vary in each individual from time to time. It may be taken, however, that any cause which results in an increase or diminution in one tissue will proportionally affect the other tissues concerned. The degree of cell response is in direct proportion to the resistance of the patient to *M. lepræ*.

For simplicity we shall first describe the cytological picture as seen in the corium. As is mentioned above, the cell most concerned in the reaction to *M. lepræ* is the endothelial cell of the capillary. Whether all endothelial cells

respond equally, or whether, as has been suggested by some writers, especial cells of the reticulo-endothelial system are chiefly concerned, it is difficult to say.

The primary and simplest reaction results in the ingestion of the bacilli by the cell. If the response is mild, the bacilli multiply in the cytoplasm of the cell, swelling it out until the typical 'foamy' or 'lepra' cell is formed. At the same time local multiplication of cells takes place, and these in turn ingest bacilli set free by the bursting of lepra cells or created by intercellular multiplication. Along with this a mucoid substance, 'gleea', is formed which cements the adjacent lepra cells and organisms into large bacillary masses.

In more resistant cases cellular multiplication is more rapid, and the cells are packed together more densely. At the same time, bacillary ingestion is followed not by multiplication of the bacilli in the cytoplasm, but by their phagocytosis and destruction. Thus, a totally different histological picture is given from that first described. Instead of the foamy bacillus-laden lepra cell, we have healthy so-called epithelioid cells, tightly packed together into cords, with a clear-cut margin surrounding the neuro-vascular plexuses, hair follicles and sweat glands. Few or no bacilli are found present in these cords.

In cases showing high resistance, the presence of a stronger cellular reaction results in the formation of giant cells, which is the indication of a still more active attempt on the part of the endothelial cells to isolate and destroy the bacilli.

#### *Results of varying degrees of cellular response in the nerve bundles.*

In the nerve bundles the reaction of cells to lepra bacilli is similar in nature to, but less in degree than, that in the corium. Also, as we have mentioned above, the bacilli are more isolated from the endothelial cells lining the capillaries in the centre of the nerve bundle. This is because of their distribution over the surfaces of the medullated fibres. For these two reasons, even in cases with high resistance, bacilli may go on multiplying for a time without phagocytosis taking place, and may form themselves into bundles and masses especially at points where there is (as mentioned above) obstruction to their upward movement in the neural lymph stream. When, however, they are at last reached by the proliferating endothelial cells, not only does phagocytosis and giant-cell formation take place in the region where the bacilli are more sparsely distributed, but also necrosis, caseation and even pus-formation result in the regions of bacillary accumulation. This may be followed by masses of bacilli being cut off from the phagocytic action of the cells; they are isolated by the caseous or purulent matter in which they are embedded and may retain their morphological features and acid-fast staining for years. This process is illustrated in Cases 1, 12, 13, 27, 37 and 81, shown in Plate VI, in Plate I, Case 12, and in Plate II, Case 10. In all of these we failed to find bacilli except where they were preserved in the caseous or purulent material.

Caseation and abscess-formation appear to result from the combination of two factors: a considerable accumulation of bacilli at one point, and high resistance of the patient to *M. lepræ* and consequently powerful cellular response.

In our experience, caseation or abscess-formation of this nature seldom occur in the skin, the reason probably being that in resistant cases phagocytosis and destruction of bacilli take place there more promptly, and thus large accumulations of bacilli are unable to take place.

We have, however, seen the sudden skin reaction which occurs in resistant cases of the neural type result in severe ulcer-formation in some three or four cases (not of this series) and Dr. Lowe has drawn our attention to the analogy between this and neural abscess-formation. This confirms the evidence that the skin and nerve lesions in these cases are essentially of the same nature, being however as a rule much more severe in nerve tissue for the reasons mentioned above.

We should mention here that abscess-formation of a somewhat different type does take place not infrequently in the skin in leprosy. In the condition commonly known as 'lepra reaction' or 'lepra fever' large cutaneous or subcutaneous nodules, containing masses of bacilli, break down and form pus, but this pus is, like that of the septic abscess, chiefly composed of polymorphonuclear and other blood cells; there are also masses of bacilli both free and contained in the cytoplasm of these cells. Whereas the pus of the neural abscess is chiefly white and homogeneous, containing few, if any, cellular elements and resembling the cold abscess of tuberculosis.

#### *Causes of varying degrees of cell response.*

We have mentioned above that local cell response is increased by the concentration of the bacilli which call it forth, a large mass of bacilli calling forth a greater local effort on the part of the tissue cells than single scattered organisms. Apart from this, there are three factors connected with varying degrees of resistance to *M. lepræ* and of cell response to the organisms in their neighbourhood. These are: the specific resistance of the patient's tissues to *M. lepræ*, the general health of the patient, and what is apparently a form of allergic reaction to *M. lepræ*. Leaving aside in the meantime the third of these three factors, we shall in the first place consider the first two, both separately and in their relationship to each other.

#### *Specific resistance or immunity to M. lepræ.*

This is shown by the degree of response elicited in certain cells of the body by the presence of *M. lepræ* in their neighbourhood. As leprosy is for practical purposes chiefly a disease of the skin, subcutaneous tissue and peripheral nerves, we shall study the reactions produced by lepra bacilli in these tissues of the body.

While blood cells also take part, especially if the reaction is strong, it will simplify matters if we confine our attention to the principal cells concerned, viz., the endothelial cells of the capillaries.

The degree of cell reaction to *M. lepræ* varies enormously in different subjects, and this variation is best made clear by contrasting a C-3 case with a typical N-1 case. Let us take for instance Case 7 (Plate IV) and compare it with Case 12 (Plates I and VI). In Case 7 we see the massive infection of the nerve, with little or no cellular reaction. In Case 12 the intense cellular reaction has led to the formation of a large nerve abscess, in which only a few bacilli could be found in the pus and none in the capsule; also the skin lesion is limited to a small patch on the ulnar side of the wrist in which no bacilli were found. In Case 7 the lesion had been noticed by the patient for only eight months. The disease had probably existed for a very much longer time, and massive infection was present in the skin all over the body. But the absence of marked reaction of the tissues, and the almost symbiotic relationship between the cells and bacilli, accounted for the absence of signs which

would attract the attention of the patient. In Case 12 the history gave a duration of three years, but as the cellular reaction to the comparatively few bacilli was intense, the signs and symptoms were marked from the beginning. It is worthy of mention that in the case of skin leprosy there was intense infection of the nerves, although neural swelling and nerve symptoms were comparatively slight; whereas in the case of neural leprosy the infection was very slight. In other words the most marked signs of leprosy are due more to the intensity of the cellular reaction to the bacilli than to the number of bacilli in the body.

We have shown elsewhere (Muir and Chatterji, 1934) that, as in tuberculosis, small infections, especially in adults, tend to raise the resistance; whereas, after a certain point, the increase of bacilli in the body lowers the resistance and the power of the cells to react to, phagocytose and destroy the bacilli. Moreover, the specific resistance of the body to *M. lepræ*, as well as to other acid-fast organisms, is especially low in the first few years of life. We cannot, however, discuss this question here at length, and our readers are referred to the article just quoted.

#### *The general health of the patient.*

The importance of the general health in influencing the genesis and course of many infectious diseases has become more widely recognized in recent years. The less toxic the causal organism of a disease, the more important does this factor become. Of the disease-causing organisms of major importance perhaps none is less toxic than *M. lepræ*. Uncomplicated leprosy, even in a marked C-3 case, does not of itself necessarily impair the general health to any marked extent, and therefore complicating factors which interfere with the general health are of extreme importance in influencing the genesis and course of the disease. Any passing condition which depresses the general health (be it a complicating disease, dietary error, or other factor), lowers temporarily the resistance of the body to leprosy infection, so that the bacilli tend to multiply in the body. Even in patients who, as the result of small leprosy infections, have acquired an enhanced specific resistance to leprosy, the temporary impairment of health will for the time being diminish or cancel that specific resistance. If health is restored in time there often follows, due to restored specific resistance, a reaction in lesions within which bacilli have multiplied during the temporary depression. Hence, the formation of the more markedly raised and erythematous skin lesions, especially those of the 'tuberculoid' type with epithelioid and giant cells. Hence also the violent cellular reactions in nerves, resulting in extreme cases in caseation and abscess-formation.

If, however, the depressing factor is too great or lasts too long, the bacilli multiply to such an extent that when health is restored the former raised specific resistance has become replaced by depressed specific resistance; in consequence the bacilli go on multiplying and the disease continues to increase.

#### *Summary of causes of caseation and abscess-formation in nerves.*

The following factors combine to cause this condition:—

- (a) Infection with *M. lepræ* of a strictly limited nature.
- (b) High specific resistance to *M. lepræ*. It is difficult to say to what extent this may be inherited; but, as we have shown elsewhere, there

is little doubt that small repeated infections with *M. lepræ* increase, at least for a time, specific resistance to *M. lepræ*.

- (c) Temporary depression or eclipse of this high specific resistance due to some passing impairment of general health. The depressing factor must not be sufficient, however, either in time or duration to allow of a very great and generalized increase of infection; otherwise the high specific immunity becomes changed into permanent low specific immunity to *M. lepræ*.
- (d) This temporary depression of health removes or impairs for the time being the power of the endothelial cells to react to the presence of, and to phagocytose bacilli. In consequence the bacilli multiply in the skin and pass up into the sensory nerve bundles and through them into the mixed peripheral nerves. In some cases the skin retains sufficient reacting power to prevent increase of organisms in its tissues, and the bacilli multiply and spread in neural tissue alone, this being more favourable than the skin for the growth of *M. lepræ*. At points of obstruction the bacilli tend to accumulate in larger or smaller masses.
- (e) Recovery from the temporary depression of health is accompanied by restoration of high specific resistance. The bacilli, which have in the meantime increased in number in the nerves, call forth a vigorous response, the 'recovery reaction', from the endothelial cells of the nerve bundle capillaries in their neighbourhood. The reaction of these cells to scattered bacilli results in the formation of a granuloma composed chiefly of epithelioid cells. Where small bunches of bacilli are present there may be giant-cell formation. Where large bunches or masses of bacilli are present there may be caseation or abscess-formation.

Case 18 (Plate VI) illustrates part of this process. Two years previously the patient had an attack of fever with rigor, probably malaria. Five months ago he first noticed the lesion on the back of his left hand. Sections of the thickened ulnar nerve branch in the neighbourhood of the skin lesion showed bacilli singly and in masses in the less granulomatous or non-granulomatous parts of the nerve, and absence of bacilli where the cellular reaction was marked. The 'leprolin' test showed that the specific resistance of the patient was only moderate in degree, hence the only moderate degree of cellular response and the absence of caseation and abscess-formation.

In Case 10, a much higher degree of specific immunity was present; but this had been temporarily lowered by an attack of kala-azar. The latter disease had been cured three months previously; and restored general health was followed by swelling of the sural nerve and the formation of a marked nerve abscess in the course of that nerve. Bacilli had disappeared, as far as our observations showed, both from the skin and from between the nerve fibres; but acid-fast bacilli were found singly and in masses, embedded in the pus of the abscess.

Several of our other cases show similarly the results of different degrees of cellular reaction; the results varying according to the specific resistance, the duration

and degree of the temporary depressing factor, and the amount and nature of neural infection that took place during the period of depression.

*The form of nerve caseation and abscess.*—When only caseation has taken place abscess generally lies more or less centrally in the nerve forming a fusiform or pea-like swelling. When pus-formation has occurred the abscess may either be central or may lie outside the nerve as a sessile sac.

In one case described by one of us elsewhere (Muir, 1924) there were three large fusiform abscesses in the course of the ulnar nerve between the axilla and the elbow. On incising the most distal of these, a large amount of white pus was evacuated; and pressure on the other two abscesses evacuated them through the incision. It was found that the stretches of nerve between the abscesses consisted of a hollow tube.

Case 12 (Plates I and VI) illustrates the lateral sessile type of abscess, pus having escaped from the centre of the nerve and ballooned out the thickened capsule.

For the sake of simplicity we have taken the nerve bundle as the unit in describing the process of abscess-formation. When, however, several parallel bundles of a composite nerve are similarly affected, caseous or purulent units will coalesce to form a large abscess. Its thick, fibrous walls may be coated with caseous material, the pus being located in the centre, as in Case 12.

The degree of impairment of nerve function varies with the amount of nerve-fibre destruction and with the degree of pressure exerted by the abscess on the functioning part of the nerve. In Case 12 there was but little loss of function, as the pus had escaped early from the centre of the nerve and exerted very little pressure on the functioning nerve fibres. In others, such as Cases 9 and 63 (Plate I), a rigid thickened capsule prevented expansion of the nerve and there was considerable pressure, and consequently anæsthesia. Excision of the nerve sheath was at once followed by restoration of more than half the lost sensation.

*Nerves as a reservoir for M. lepræ.*—We have shown above that in the nerves *M. lepræ* are more protected from the phagocytic action of cells than they are in the skin. We have also suggested that this may, at least in part, be due to their sheltered position between the medullated nerve fibres at a distance from the endothelial cells of the capillaries. We have also referred to the fact that, while small infections with *M. lepræ* increase the specific resistance, large infections lower the resistance, or rather produce specific tolerance to this organism. The massive infection in C-2 and C-3 cases, with but little proportionate cellular reaction, noticeable in the skin but even more marked in the nerves, and the negative results with the leprolin test in these cases, give strong evidence of the truth of this view.

There is, therefore, considerable likelihood that the peripheral nerves form the principal reservoir for *M. lepræ* in the body, at least in incipient cases of leprosy. Between the nerve fibres they can multiply, and, in cases with little or even moderate specific or general resistance, they escape any marked control by the phagocytic action of the cells. In the neural tissue they may thus multiply until they reach a number sufficient to produce specific tolerance. Thereafter, the phagocytic action of the cutaneous cells is lowered, and multiplication and spread of the infection becomes possible in the skin. Thus initial neural infection becomes transformed into cutaneous leprosy.

It is difficult to do more than surmise as to how the bacilli pass from one nerve to another. It would seem that escape from a nerve must take place by way of the skin, connective tissue or lymph nodes. We have made careful sections of lymph nodes in many cases of initial and limited nerve leprosy and have uniformly failed to find either bacilli, or signs of cellular reaction which would indicate their having been present. As we have seen above, the cells of both the skin and neural connective tissue react more readily to the presence of bacilli than do those of the nerve bundles.

When, therefore, there is adequate resistance, the bacilli tend to be sealed up inside the infected nerve by the surrounding connective tissue and the distal skin. In evidence of this is the remarkable fact that in the great majority of our cases the disease is confined to the one or two nerves originally infected from the skin lesion in their sensory distribution. While in some cases a marked neural infection has existed, it may be for years, in the one or two nerves involved, the rest of the body has escaped. When, however, resistance is lowered sufficiently to break the seal and make the skin filter pervious, the infection finds its way out through the perineural connective tissue, and other nerves are liable to become infected.

An illustration of the way in which infection may spread through the subcutaneous plexus in the periphery of a leprosy lesion of the skin was shown in some of our sections. As the lesion extended, papules appeared at a short distance beyond the margin. Sections through the margin including a papule and the intermediate healthy skin showed the spread of infection in the subcutaneous plexus passing beyond the original lesion and spreading up the hair follicles to the subpapillary plexus and the papillæ. From there it spread directly through the superficial layers of the skin. Thus the ultramarginal papule became continuous with the advancing lesion. As nerves and vessels are closely mixed together in the perifollicular region, it was impossible to state that the infection passed up to the surface in neural as opposed to vascular tissue, but in the subcutaneous layer the infection was distinctly intraneural. The infection might be compared to a plant like the sweet potato, spreading underground and sending up its shoots to the surface at short intervals.

In Case 4 (Plates V and VI) we have an example of a lesion at first apparently healed and then beginning to spread at two points on the margin of the apparently healed lesion. Sections of the thickened supplying nerve branch showed caseation due to a former *recovery reaction*, but bacilli were found among the nerve fibres outside the caseous area. Apparently the *recovery reaction* had not been sufficient entirely to destroy the bacilli in the nerves, and a subsequent depression of general health allowed the bacilli once more to pass down from the nerve bundles and invade the skin.

In Case 54 we have a somewhat similar condition. The former extensive lesion covering the whole forearm has apparently healed. In the highly granulomatous nerve branches also the bacilli have been eradicated. The skin lesion showed signs, however, of gradual proximal spread, and bacilli were found between the nerve fibres in the non-granulomatous areas of the bundles. This case suggests re-infection of the skin from the nerve branches during a period of depressed general health.



*Local and distal symptoms of neural origin.*—We have referred above to the non-toxic nature of leprosy and the fact that even in a C-3 case the general health may be excellent. The most distressing symptoms are connected more with neural than with cutaneous leprosy. We have already mentioned the different forms of caseation and nerve abscess and shown that pain, tenderness, anæsthesia and other symptoms are due to pressure caused by cell proliferation, caseous material and pus, and that these symptoms are most severe in cases with very marked central inflammation accompanied by a rigid fibrous capsule which prevents expansion, the fibres being pressed between the swelling interior and the rigid exterior. We have shown, especially by reference to Cases 9 and 63 (Plate I), the effects of removal of the nerve sheath in such cases in reducing anæsthesia, pain and tenderness; and the relief of pain, etc., after opening nerve abscesses.

We would call attention also to the partial restoration of sensation after excision of portions of granulomatous nerve branches in Cases 21, 47, 59 and 83 (Plates II and VI).

Anæsthesia, as found in nerve leprosy, may be divided into two types: primary and secondary. Primary anæsthesia is due to the destruction of small nerve branches in the skin and subcutaneous tissue. Secondary anæsthesia is due to the infection passing up into the larger sensory and mixed nerves and causing pressure upon bundles of fibres which supply healthy skin. Removal of the nerve sheath and the opening of nerve abscesses cause restoration of the latter and not of the former. The diminution of anæsthesia after excision of small diseased branches may be due to the surrounding healthy nerve branches taking up the function of the excised diseased branches. The restoration is too rapid to be explained by growth of axicylinders from the proximal ends of the excised nerves.

*Allergic reaction.*—*Lepra fever*, otherwise known as *lepra reaction*, is a condition in which there is sudden swelling up of leprosy lesions and appearance of new lesions along with febrile and other general disturbances. It is found chiefly in fairly advanced and progressive cases of the cutaneous type, and generally occurs in patients with poor general health, or in those who are suffering from other accompanying diseases. It may be of a continuous or intermittent type. The sudden and repeated swelling up and subsiding of lesions is not accompanied by a proportionate increase and diminution of *M. lepræ* in these lesions.

There is good reason, in our opinion, for applying the term 'allergic' to this latter form of reaction. Whereas the 'recovery reaction' described above does appear to be as a rule of an allergic nature; it is not a hypersensitization, but a restoration of the temporarily depressed power of the cells to react to *M. lepræ*. While typical cases of each type of reaction are easily distinguished from one another, there are some cases in which the one form may appear to merge into the other.

As mentioned above, in severe reactions of the 'lepra fever' type, cutaneous nodules may break down and give rise to acute abscesses full of pus cells and bacilli, the cells being chiefly phagocytic blood cells. There may also be sudden, painful swelling up of infected nerves. In the case of the ulnar nerve there may be severe permanent impairment of the small muscles of the hand unless pressure is relieved by decapsulation without delay.

Case 8 (Plate I) is a remarkable example of small localized abscesses in a C-2 N-2 case. An abscess of the medial cutaneous nerve is shown, and four scars of previous abscesses are also visible. We find it difficult to classify this case, as the lesions are similar to those found in *recovery reaction*, while the widespread skin infection and the prolonged febrile symptoms point to the *lepra fever* type.

We must confess, however, that little is yet known of the nature of lepra fever; and terms like 'allergy', which have been applied in the study of diseases of a much more toxic nature, should be used with caution in describing leprosy.

### SUMMARY.

1. Bacilli enter and pass up nerve branches from the neuro-vascular plexuses of the skin. This may, or may not, be accompanied by clinical skin lesions. Either the local branches, or the distant main nerves, or both, may show clinical changes. Bacilli lie in close connection with neuro-vascular plexuses in the skin and subcutaneous tissue. The nerve bundles form a specially favourable medium for the growth of *M. lepræ*.

2. Bacilli are carried up the nerves from the skin by the lymph flow. They tend to accumulate at points of obstruction.

3. The 'neurotropism' of *M. lepræ* is discussed. This is shown to be at least partly connected with the comparative absence of cellular response to the bacilli in the nerves.

4. The endothelial cells of the capillaries take the most important part in the response to *M. lepræ* in both the skin and the nerves. In the latter the bacilli are isolated from the endothelial cells of the centrally placed capillary by the medullated nerve fibres, and thus may escape phagocytic destruction. In the skin the bacilli lie in close proximity to the endothelial cells, and are thus more liable to be phagocytosed.

5. The effects of varying degrees of cellular reaction to lepra bacilli in the skin and in the nerves are discussed, especially with regard to the formation of nerve abscess.

6. Apart from the degree of local concentration of bacilli, there are three factors which account for varying degrees of resistance and cellular response to *M. lepræ*, viz., specific resistance, general health and allergic reaction.

7. The course of events is traced in the formation of caseation and nerve abscess; the forms which nerve abscesses assume are described and discussed in relation to their effect on nerve functions.

8. The nerves form an important reservoir for bacilli where they may multiply until sufficient tolerance is produced to overcome the greater resistance in the skin. This is followed by spreading of the bacilli to other nerves, and frequently by generalized infection.

9. Pain and other symptoms are relieved in suitable cases by excision of the nerve sheath of thickened nerves, opening of abscesses and excision of diseased nerve branches. A distinction is made between primary and secondary anæsthesia.

10. 'Recovery reaction' is distinguished from allergic reaction.

and epidemic form. Only four cases of typhus were admitted to hospital during 1935 from among the British troops in the area, two from Solan, one from Dagshai and one from Kasauli. A clinical account of these cases, and also of two Indian cases treated at the Cantonment General Hospital at Kasauli, has been given by Bush (1936).

It has been the experience of workers in other countries that in order to establish typhus strains from human sources the most favourable time for taking the patient's blood is within the first three or four days from the first onset of fever, i.e., before it is possible to obtain a positive Weil-Felix reaction, or to make a clinical diagnosis with certainty. Arrangements were, therefore, made to take blood from any patient with fever admitted to hospital in Kasauli as soon as a preliminary test for malaria parasites had proved negative. Unfortunately, all the cases of fever where blood was taken early in the disease subsequently proved not to be typhus, and on only three occasions was blood obtained from genuine typhus cases before the fever had subsided. In each case the Weil-Felix reaction given by the patient's serum was positive with *Proteus* OXK and negative with OX19 and OX2\*. An account of the attempts made to establish typhus strains from these three cases is given below. No opportunity arose for obtaining blood from a human case whose serum agglutinated OX19 :—

(1) Case GK was a British soldier. The highest Weil-Felix reaction recorded was 1 : 200 with OXK on the 22nd day of the disease. Blood taken on the 11th day was defibrinated, and inoculated intraperitoneally into two guinea-pigs, GK/1 and GK/2, each of which received about 5 c.c. GK/1 developed a temperature of 103.0°F. on the fourth day, and was found dying on the fifth. Sub-passage was made with spleen emulsion into guinea-pigs GK/3 and GK/4, both of which died within 24 hours. GK/2 did not develop fever, but lost weight rapidly from the fourth day, and was found in a dying condition on the eighth. Sub-passage was made with spleen emulsion intraperitoneally into guinea-pigs GK/5 and GK/6, but both were found dead next morning, and the strain was lost. Post-mortem examination of these animals showed no special lesions except congestion of the spleen, and in one case hæmorrhagic foci in the lungs. Cultures of the blood were not taken, but the symptoms noted were similar to those which occurred in some of the guinea-pigs used in sub-passaging strains RD and WL, and which were proved to be due to infections with *B. ærtrycke*. It is considered probable that the deaths of the six guinea-pigs used in attempting to establish strain GK were also due to secondary infections with this organism.

(2) Case RD was an Indian of the poorer class, who had been employed as a cook. The highest Weil-Felix reaction recorded was 1 : 5,000 with OXK on the 11th day of the disease. This patient developed broncho-pneumonia, and died on the 22nd day from the first onset of fever. Blood taken on the 9th day was defibrinated, and inoculated intraperitoneally into two guinea-pigs. Each of these animals developed a moderate febrile reaction, commencing on the 9th and 10th days after infection respectively. The strain was carried on through four generations by intraperitoneal inoculation of spleen or brain emulsion, one or more animals in each sub-passage developing a mild febrile reaction, which commenced about the

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\* All Weil-Felix tests referred to in this paper were carried out with concentrated alcoholized suspensions of *Proteus* X strains.

8th to 10th day. None of the three animals inoculated at the fifth sub-passage showed any reaction, and the strain was lost. None of the passage animals showed any outward sign of a scrotal reaction, although in some cases thickening and injection of the tunica vaginalis with increased exudate was noted post mortem. Organisms resembling *Rickettsia* were seen in scanty numbers in scrapings from the tunica vaginalis of certain of the passage animals. Two of the 19 guinea-pigs used for sub-passaging this strain died within the first few days following inoculation, with symptoms similar to those noted above in the case of strain GK. A culture taken from the heart-blood of one of these resulted in a pure growth of *B. ærtrycke*.

Histological examination of the brain of one of the guinea-pigs of the third sub-passage revealed the presence of a number of lesions resembling those described by other workers as typical of infection with typhus. This animal had developed a febrile reaction on the 11th and 12th days after infection, and again on the 15th to 19th days, and was killed on the 21st day. Sections of the brain showed proliferative lesions associated with the smaller blood vessels. Various stages were seen, varying from a concentration of one or two layers of neuroglia cells round a capillary to larger infiltrations resulting in the formation of a nodule, with more or less complete obliteration of capillaries. Small perivascular hæmorrhages were also present. The lesions, which were most numerous in the cerebral cortex, appeared to resemble very closely those described by Lewthwaite (1936) in his account of the histological changes met with in the brain in tropical (rural) typhus.

(3) Case WL was a British soldier. The highest Weil-Felix reaction recorded was only 1 : 50 with OXK, on the 22nd day of the disease, but clinically the case was considered to be typical of typhus. Blood taken on the 10th day was defibrinated, and inoculated intraperitoneally into two guinea-pigs. One of these developed a mild febrile reaction on the 8th day, and the strain was maintained by sub-passage of spleen or brain emulsion intraperitoneally in guinea-pigs through 7 generations, one or more animals in each sub-passage developing a febrile reaction which commenced about the 8th to 10th day, as in the case of strain RD. The fever was of a mild type, seldom exceeding 103·0°F. Most of the reacting animals were killed for sub-passage, and the full course of the pyrexia was observed in six cases only. In these, the initial fever lasted only two or three days, but in four of the cases there were irregular rises of temperature for short periods during the second and third weeks after inoculation. In the majority of cases there was no loss of weight, but a few animals lost about 10 per cent of their body-weight during the initial three days of fever. At the eighth sub-passage, none of the four animals inoculated showed any reaction, and the strain was lost. A definite scrotal reaction was observed in one guinea-pig at the fourth sub-passage, though it was less marked than that regularly noted in guinea-pigs inoculated with strains recovered from wild rats. As in the case of strain RD, organisms resembling *Rickettsia* were occasionally seen in scanty numbers in scrapings from the tunica vaginalis of passage guinea-pigs and white rats. During the course of these experiments, 16 guinea-pigs out of 31 used developed high fever and died during the first few days after inoculation. Cultures taken from the heart-blood of three of these animals yielded a pure growth of *B. ærtrycke* in each case, and it is considered probable that the deaths of all these animals are attributable to secondary infection with this organism.

Attempts were made to establish strains RD and WL in rabbits by inoculating passage virus into the anterior chamber of the eye, by the method practised successfully by Nagayo and his colleagues (1931) in Japan in the case of tsutsugamushi disease, and by Lewthwaite and Savor (1932) in Malaya in the case of rural typhus. An irido-cyclitis was produced in several animals, but attempts to carry on the strain in further passages were unsuccessful, except in one instance. Four rabbits had been inoculated intra-ocularly with spleen emulsion from a reacting guinea-pig of the fifth sub-passage of strain WL. One rabbit developed an irido-cyclitis, and the strain was carried on through four generations by intra-ocular inoculation of aqueous humour. Only about 20 per cent of the inoculated animals showed any reaction, however, and the strain was lost at the fifth intra-ocular sub-passage, three months after the primary inoculation of the patient's blood into guinea-pigs. Scrapings from Descemet's membrane were examined in the case of several of the reacting rabbits, but in spite of careful search, *Rickettsiæ* were not observed.

On two occasions passage virus from strain WL was inoculated into monkeys [*Silenus (Macacus) rhesus*]. In the first experiment two monkeys, WL/1 and WL/2, each received an emulsion containing half the brain of a passage guinea-pig of the fourth generation subcutaneously. Monkey WL/1 developed a temperature of 103·4°F. on the fifth day, and the fever continued until the 10th day, after which it did not exceed 102·4°F. The highest temperature recorded was 104·9°F. on the 9th day. Monkey WL/2 developed a temperature of 103·0°F. on the 7th day, and the fever continued until the 11th day, after which the temperature did not exceed 102·4°F. The highest temperature recorded was 103·3°F. on the 9th day. The reaction produced in these two animals resembles that which followed the inoculation of one of our rat strains into a monkey, which showed a rise of temperature on the 8th, 9th and 10th days after infection (Covell, 1936b).

In the second experiment, three monkeys were inoculated subcutaneously with material derived from a passage guinea-pig of the 5th generation. Monkey WL/3 received an emulsion containing one-third of the spleen, monkey WL/4 received 4 c.c. of defibrinated heart-blood, and monkey WL/5 received an emulsion containing one-half of the brain. The first two animals showed no rise of temperature during an observation period of three weeks, but monkey WL/5 developed a temperature of 103·0°F. and 104·0°F. on the 15th and 16th days after inoculation respectively. None of the animals used in either experiment showed any definite symptoms of illness, and in no case was the result of the Weil-Felix test significant, the highest agglutination titre recorded being 1 : 70 with OXK.

The Weil-Felix test was carried out with the sera of a number of rabbits which had been inoculated either intraperitoneally or intra-ocularly with passage material from strains RD or WL. Out of 29 rabbits on which the test was performed at five-day intervals over a period of 40 days, 6 gave some degree of agglutination with OXK (Table I).

Though the agglutination titres were low, the waxing and waning curves are considered to be suggestive. The results given by the serum of WL/28 are interesting as showing the late development of agglutinins for *Proteus* strains in the rabbit, to which Felix (1933) has drawn attention. None of the sera gave any significant degree of agglutination with OX19 or OX2.

Eighteen white rats were inoculated intraperitoneally with passage material from strains RD or WL. Organisms resembling *Rickettsia* were observed in scrapings made from the tunica vaginalis of some of these animals, but in scanty numbers only. The temperatures of the rats were not recorded. The sera of 12 of the animals were tested for the Weil-Felix reaction, and agglutination was observed in two cases with OX19 in a dilution of 1 : 50, a finding which will be referred to later. There was no significant result with OXK.

TABLE I.

*Results of Weil-Felix tests in rabbits with OXK.*

Days after inoculation.	NUMBER OF RABBIT.					
	RD/4.	RD/6.	WL/2.	WL/7.	WL/20.	WL/28.
0	0	0	25	0	0	25
10	0	25	125	0	0	0
15	0	45	125	17	50	0
20	25	25	70	15	27*	0
25	50	0	60	25	..	0
30	35	0	0	15	..	55
35	17	0	..	0	..	70
40	15	0	..	0	..	85

\* WL/20 was killed for passage on the 21st day after inoculation.

As regards cross-immunity experiments, 10 guinea-pigs previously inoculated with passage virus from one or other of the human strains all reacted in a typical manner when inoculated with one or other of the rat strains. On the other hand, out of 8 guinea-pigs which had previously reacted to infection with one or other of the rat strains, only one showed a febrile reaction following inoculation with a human strain. These experiments were carried out, however, when the human strains were apparently weakening, and when only a small proportion of normal guinea-pigs were showing a febrile reaction to them. It is considered that such evidence as is available indicate that there is little if any cross-immunity between the human and rat strains.

#### DISCUSSION.

The results of the experiments detailed above show that the clinical and serological reactions produced in laboratory animals by our rat strains differ markedly in certain respects from those which follow the inoculation of strains derived from human cases of typhus.

The rat strains produce a severe febrile reaction in guinea-pigs accompanied regularly by an acute specific orchitis, and typical intracellular *Rickettsia* are demonstrable in abundance in scrapings from the tunica vaginalis. Two strains have been maintained in these animals for 9 months in the laboratory without difficulty. In white rats, though there is no outward sign of a scrotal reaction, typical intracellular *Rickettsia* are found in large numbers in scrapings from the tunica vaginalis of infected animals. The sera of white rats and rabbits infected with the rat strains have yielded a markedly positive Weil-Felix reaction with *Proteus* OX19 in a considerable proportion of cases.

With the human strains, on the other hand, a febrile reaction in passage guinea-pigs occurred less frequently than with the rat strains, and, where present, it was of mild degree only. A scrotal reaction was of rare occurrence, and in no case has a reaction been observed which was comparable in severity with that regularly produced by the rat strains. It has not been found possible to maintain human strains in guinea-pigs beyond the 4th sub-passage in one case and the 7th in another. Organisms resembling *Rickettsia* have been found in scanty numbers only in scrapings from the tunica vaginalis of passage guinea-pigs, and these were not sufficiently typical to warrant a certain diagnosis in the absence of other evidence. In white rats, the same remark regarding *Rickettsia*-like organisms applies; whilst in both white rats and rabbits the response to the Weil-Felix test, if present at all, was observed in comparatively low dilution only.

The results of our attempts to establish strains of typhus from human sources, though disappointing, were not altogether unexpected, in view of the experiences of other workers.

Shortt and D'Silva (1936) have described experiments carried out with material obtained from human cases of typhus in Kasauli in 1934. As in the experiments detailed above, the sera of all the cases had given positive Weil-Felix reactions with *Proteus* OXK only, and the blood was in no case taken in the very early stages of the disease. Evidence of a definite infective process after inoculation of laboratory animals was limited to those which received direct intraperitoneal inoculation of patients' blood. A mild febrile reaction was noted in only two out of 28 guinea-pigs used, and in no case was a scrotal reaction observed. Six out of 8 white rats inoculated with patients' blood reacted with fever, and organisms resembling *Rickettsia* were seen in scrapings from the tunica vaginalis of guinea-pigs and white rats. One rabbit out of three gave a positive Weil-Felix reaction with *Proteus* OXK. The serological results in the case of the white rats were of interest, in view of the findings recorded by Anigstein (1933) with the sera of passage rats (*vide infra*). Out of 8 sera tested, 5 gave some degree of agglutination with OX19, the highest titres being 1 : 125 in one case, and 1 : 50 in two others. It has been our experience that whilst the sera of normal white rats very frequently show standard or total agglutination with OXK in a dilution of 1 : 50, it is rare for any agglutination to occur with OX19, even in a dilution of 1 : 25.

In Malaya, similar difficulty has been experienced in establishing strains from human sources. Fletcher and Lesslar (1925) inoculated a number of guinea-pigs with the blood of typhus patients, and two animals reacted with fever, but the strain was lost after the second sub-passage. Lewthwaite (1930) inoculated 113 guinea-pigs with the blood of rural typhus patients or with material from passage

animals, but only 7 developed fever, and in no case was a scrotal reaction observed. Attempts to maintain strains beyond the third sub-passage in guinea-pigs and rats failed. In rats, 25 developed a febrile reaction out of 87 inoculated from 62 patients.

Anigstein (*loc. cit.*), also working in Malaya, obtained a febrile response in 11 per cent out of 440 guinea-pigs inoculated with virus obtained directly or indirectly from human cases of typhus. In the case of one strain, which was derived from a fatal case of rural typhus and which was maintained for 11 generations, a scrotal reaction was observed in 5 guinea-pigs out of 67 used; in experiments with another strain, this reaction was noted in only one case out of 27. In inoculated rats, 35 per cent gave a positive Weil-Felix reaction, 20 per cent reacted with fever, and 12 per cent showed some degree of scrotal reaction. Anigstein notes that the Weil-Felix reaction in rabbits was inconstant, and of a lower titre than that recorded in rats. It is interesting to note that the sera of two rats inoculated with passage virus agglutinated suspensions of *Proteus* OX19, despite the fact that the original virus belonged to a K strain, as has been noted above in the case of certain white rats used in Shortt and D'Silva's experiments and in our own. Additional significance is given to these findings by the result of an experiment of Anigstein, in which a transformation of the XK to the X19 form apparently occurred in the rat, and was transmitted in this form through the guinea-pig to man. It is noteworthy also that Lewthwaite and Savor (1932) have established a typhus strain from wild rats which produces agglutinins sometimes for OX19 and sometimes for OXK.

In only one instance in Malaya have the numerous attempts made to establish a strain of rural typhus (i.e., K form) in guinea-pigs or rats from human sources succeeded, in spite of the relatively abundant clinical material available. This strain was derived from blood drawn from a patient early in the disease and inoculated into guinea-pigs which had been fed on a vitamin-deficient diet (Lewthwaite and Savor, 1933). Passage guinea-pigs have reacted with fever, but never with scrotal swelling, and the Weil-Felix reaction in rabbits inoculated with passage virus has on various occasions been positive with OXK, but never with OX19. A strain of urban typhus (i.e., agglutinating *Proteus* OX19) has also been successfully established in guinea-pigs by these authors. This strain produces a scrotal reaction in the majority of passage guinea-pigs.

It was on account of the difficulties encountered in maintaining strains of rural typhus in guinea-pigs and rats that Lewthwaite and Savor (1932) attempted to establish strains in rabbits by the intra-ocular method of Nagayo. These attempts were successful, and it has been found possible to initiate and maintain strains of rural typhus without difficulty, after the first two or three sub-passages have been successfully negotiated. Our own failure to establish strains of typhus in rabbits from human sources by the intra-ocular method has been a great disappointment. It must be remembered, however, that an opportunity has not yet occurred to obtain blood for this purpose direct from a patient in the early stages of the disease, and it is felt that this method offers the best prospect of success in future experiments.

#### NOTE ON THE STAINING OF *Rickettsiæ* IN SMEARS.

It is generally agreed that the best method of demonstrating *Rickettsiæ* in smears is by the use of Giemsa's stain, but various modifications in technique have



been suggested. The writer has obtained satisfactory results in the case of scrapings from the tunica vaginalis of rats and guinea-pigs by the following method: The smear, after thorough drying but without preliminary fixation, is treated with Giemsa's stain made up in the proportion of 20 drops of stain to 15 c.c. of distilled water for 4 hours. The stain is flushed off with distilled water, and the slide allowed to dry. It is then rinsed in xylol and afterwards passed rapidly in succession through absolute alcohol, 90 per cent alcohol, 50 per cent alcohol and 15 per cent alcohol. It is finally washed in distilled water, and allowed to dry.

#### NOTE ON THE TECHNIQUE OF THE WEIL-FELIX TEST.

During the past few months, 1,260 sera have been examined by the Weil-Felix test with concentrated alcoholized suspensions of both *Proteus* OX19 and *Proteus* OXK, and in a number of cases with *Proteus* OX2 also. In carrying out these tests, much time and labour have been saved by adopting the technique advocated by Bridges (1935). In this method the Dreyer's tubes (after the addition of serum) are filled rapidly to approximately half an inch from the brim with normal saline, instead of measuring the latter by drops from a pipette. As Bridges has shown, the error thus introduced is so slight as to be negligible. We have, however, slightly modified the procedure as follows: Before the tubes are taken into use, 24 drops of saline are dropped into each from a Dreyer's pipette, and a mark is made with a diamond at the upper level of the fluid. In performing the test, saline is poured into each tube up to the mark (after the addition of serum), before the single drop of concentrated suspension of *Proteus* X is added.

#### ACKNOWLEDGMENTS.

I wish to express my thanks to Dr. R. Lewthwaite, who in a personal communication to the writer very kindly described in detail the technique of his intra-ocular operation; to Lieut.-Colonel R. F. Bridges, R.A.M.C., Officer-in-Charge, Enteric Laboratory, Kasauli, for supplying the concentrated alcoholized suspensions of *Proteus* X strains used in the Weil-Felix tests, and for examining a number of cultures from passage guinea-pigs; to Captain F. K. Bush, R.A.M.C., for allowing me access to the patients under his charge at the British Military Hospital and Cantonment General Hospital, Kasauli; and to Sub-Assistant Surgeon B. N. Lahiri, I.M.D., of the Pasteur Institute of India, for his help in carrying out numerous animal passages.

#### SUMMARY.

An account is given of attempts to establish typhus strains from human sources in laboratory animals.

Two strains were maintained in guinea-pigs for four and seven generations respectively, and the latter was further carried on for four sub-passages in rabbits by the intra-ocular method, after which it was lost. The various reactions produced by the strains in laboratory animals are described.

Although the attempts to establish typhus strains from human sources ended in failure, they are recorded because it is thought that an account of the experiments carried out may be of assistance to other workers in India.

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## THE RÔLE OF MALARIA IN THE CAUSATION OF CIRRHOSIS OF THE LIVER.

BY

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In a previous communication (Tirumurti and Radhakrishna Rao, 1935) we pointed out that the common association of malaria and cirrhosis of the liver in India has given rise to divergence of opinion regarding the rôle of the former in the causation of the latter. This problem is an important one in India on account of the frequency with which malaria is associated with cirrhosis of the liver (Hughes and Shrivastava, 1927 ; Hughes, 1933 ; Hameed, 1933 ; Radhakrishna Rao, 1933) and the ease with which the latter could be eradicated in time, if the former could be proved to be connected with the latter as cause and effect, by early detection and a more thorough treatment of malaria.

We have already pointed out (Tirumurti and Radhakrishna Rao, *loc. cit.*) that in our experience malaria *per se* is not a direct cause of cirrhosis of the liver. But on account of the importance of the problem and the divergent views regarding the same, we reinvestigated the whole question by improved staining methods and the results form the basis of this communication.

### MATERIAL AND METHODS OF STUDY.

As a correct understanding of the problem under discussion depends on an accurate study of the nature and genesis of fibrosis, if any, in chronic malarial livers, we bestowed our attention on this line of investigation. In addition to our own collection of chronic malarial livers in the Pathology Museum of the Vizagapatam Medical College, we also investigated the autopsy material (chronic malarial livers) obtained from the Madras Medical College and the Stanley Medical School, Madras,

through the courtesy of Dr. A. Vasudevan and Dr. T. Bhaskara Menon respectively. Briefly our method of investigation was as follows :—

From each of the specimens of chronic malarial livers several thin pieces were taken so as to include in the parenchyma the different orders of the divisions of the portal and hepatic venous trees, to study the changes, if any, at the different levels of the vascular and biliary trees. The pieces of the liver were fixed in 10 per cent neutral formalin. In the case of old museum specimens, the pieces were first washed in running tap-water in a histological washing tank for 24 to 48 hours and then fixed in 10 per cent neutral formalin. For routine examination paraffin sections were stained with Ehrlich's acid hæmatoxylin and eosin : for staining the connective tissue, the paraffin sections were stained with Weigert's iron-hæmatoxylin and van Gieson stain. Frozen sections were stained with the Foot and Ménard's (1927) silver carbonate impregnation method to bring out the reticulum of the liver. It may be mentioned here that this method of staining the reticulum of the liver has given very satisfactory results in our hands.

#### REPORT OF INVESTIGATION.

The macroscopical and microscopical appearances in the liver in the material under investigation could be understood from the brief descriptions of some of the typical cases given below :—

*Case I (Plate VII).*—The liver weighed 40 oz.; surface smooth; dark brownish in colour; dark brownish on section; lobular markings distinct; the organ cuts with resistance; consistency firmer than normal; Prussian-blue reaction positive.

*Microscopically*—no loss of lobular pattern; sporadic fatty change; the sinusoids contain Kupffer cells loaded with malarial pigment; no fibrosis; endo-phlebitis of a collecting vein.

*Case II.*—The liver weighed 40 oz.; normal in size; surface slightly uneven; capsule thickened in places; section dry and of a deep brownish colour; cuts with resistance and very firm; Prussian-blue reaction positive.

*Microscopically*—slight thickening and irregularity of the capsule; slight condensation of the reticulum around the central veins; no loss of lobular pattern; sporadic fatty change; sinusoids prominent and empty; Kupffer cells loaded with malarial pigment; slight widening of the portal spaces. Groups of hepatic cords, especially in the centre of the lobule, were separated from each other by intralobular œdema, giving rise to a false impression of cirrhosis, but there is no typical pseudo-lobulation.

*Case III.*—The liver weighed 46 oz.; surface dark brownish in colour; normal in size; capsule thickened; firm in consistency and cuts with resistance; section dark brown in colour; lobular markings indistinct.

*Microscopically*—no loss of lobular pattern; no increase of fibrous tissue in any of the ramification of the Glisson's capsule; the sinusoids were plugged with dark brown pigment, which is especially seen in the Kupffer cells.

*Case IV (Plate VIII).*—The liver weighed 48 oz.; slightly enlarged in size; the capsule thin; the surface greyish in colour; cut section moist and slaty in colour; lobular markings indistinct.

*Microscopically*—no fibrosis in the organ; Kupffer cells loaded with malarial pigment; no loss of lobular pattern.

*Case V.*—Chronic malarial liver (Stanley Medical School specimen).

*Microscopically*—sinusoids congested; Kupffer cells loaded with dark malarial pigment; sporadic fatty change; no loss of lobular pattern; fibrosis not marked; slight widening of the portal spaces; focal necroses of the hepatic cords.

*Case VI.*—Chronic malarial liver (Stanley Medical School specimen).

*Microscopically*—no loss of lobular pattern; slight widening of the portal spaces; hepatic cords contracted but in places show cloudy swelling; Kupffer cells contain dark brown malarial pigment in moderate amount; slight pericholangitic fibrosis.

PLATE VII.

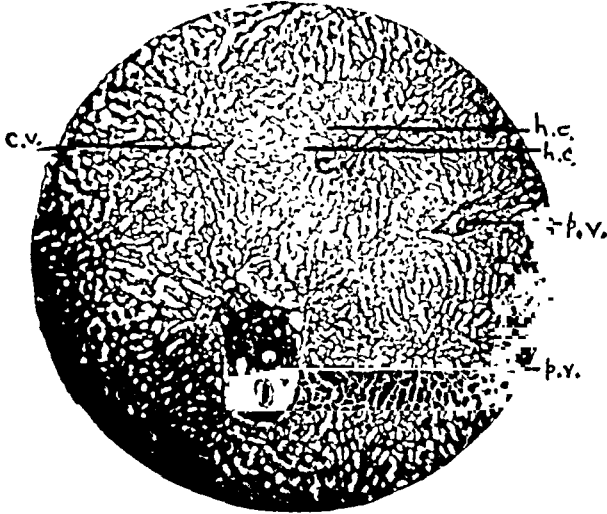


FIG. 1. *Case I.*—Photomicrograph of the reticulum of the liver showing the normal pattern of the hepatic parenchyma and the absence of fibrosis. The hepatic cords (*h.c.*) are unstained and are represented by the empty spaces in the figure.

*p.v.*—portal space; *c.v.*—central vein. (Foot and Ménard's silver carbonate impregnation technique.)



FIG. 2. *Case I.*—Photomicrograph of the section of the liver showing the sinusoids which contain Kupffer cells (*a*) loaded with malarial pigment.

*h.c.*—hepatic cords. (Ehrlich's acid hæmatoxylin and eosin.)

PLATE VIII.

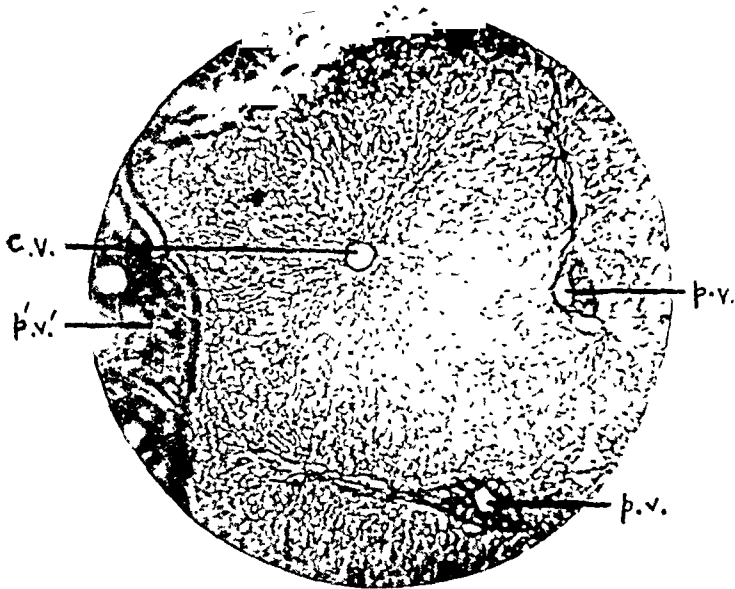


FIG. 3. *Case IV.*—Photomicrograph of the reticulum of the liver showing the normal architecture of the hepatic parenchyma and the absence of fibrosis. The hepatic cords are unstained and are represented by the empty spaces in the figure.

*p.v.*—small portal space; *p'.v'*.—portal space of the third order; *c.v.*—central vein. (Foot and Mênard's silver carbonate impregnation technique.)

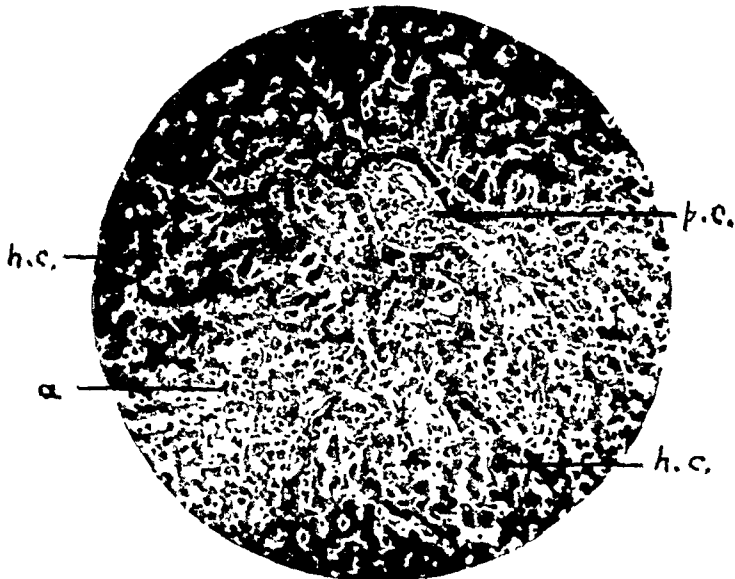


FIG. 4. *Case IV.*—Photomicrograph of the section of the liver showing the Kupffer cells (*a*) loaded with dark brown malarial pigment.

*p.c.*—portal space; *h.c.*—hepatic cords. (Ehrlich's acid hæmatoxylin and eosin.)

It may be noted from the above description that the *important histopathological features* in the liver in this series are : absence of any loss of lobular pattern ; loading of Kupffer cells with malarial pigment ; and no noticeable increase of fibrous tissue in the liver. Sporadic fatty degeneration and focal necroses of the hepatic cords, slight sclerotic widening of the portal spaces and congestion of the sinusoids were some of the other, but inconstant, findings. Endo-phlebitis of the collecting veins of the hepatic venous tree and pericholangitic fibrosis were occasional and rare findings. *In none of the cases investigated was there any pseudo-lobulation or fibrosis similar to that seen in a typical case of cirrhosis of the liver.*

### DISCUSSION.

The indiscriminate use of the term 'cirrhosis' to any and every type of fibrosis of the liver, has no doubt added to the confusion regarding the rôle of malaria in cirrhosis of the liver. The term 'cirrhosis' was first invented by Laennec in 1819 to denote the condition of portal cirrhosis (now named after him) in which the following features stand out prominently : (1) necrosis of the hepatic parenchyma ; (2) replacement (residual) fibrosis ; (3) atypical regeneration of the hepatic parenchyma (pseudo-lobulation) and (4) involvement of the whole organ. Elsewhere (Ramachandra Rao, 1933 ; Radhakrishna Rao, 1935*a* and *b*) ; Ramachandra Rao and Radhakrishna Rao, 1934) it is pointed out that the word 'cirrhosis' should be limited to an organ showing all the above-mentioned features. Though it is out of place here to discuss the definition and classification of cirrhosis of the liver, we wish to point out that the word 'cirrhosis' should only be used to the portal and toxic types of hepatic fibrosis, as these alone exhibit all the features mentioned above.

Judging from the above standards, none of the cases studied in this series showed a true cirrhotic condition of the liver. None of the specimens exhibited any loss of lobular pattern. Except slight widening of the portal spaces, the fibrosis in the liver was negligible. Though in all the instances the Kupffer cells were loaded with malarial pigment, the silver impregnation of the reticulum of the liver showed absence of marked fibrosis. In some of the hæmatoxylin and eosin stained sections, a division of the parenchyma into small, irregular islands by intralobular œdema simulated pseudo-lobulation ; but the silver impregnation of the reticulum of the same specimen showed only a normal hepatic architecture.

As pointed out by Rolleston and McNec (1929), the possibility of slight necrosis of the hepatic parenchyma in acute or sub-acute malaria cannot be denied, but the liver possesses a remarkable capacity to regenerate (Mann and Bollman, 1926) so that such necrotic changes leave no permanent trace behind. In almost all the instances in this series, the chronic malarial liver showed no increase of fibrous tissue.

The causes of cirrhosis of the liver are so many that each case should be thoroughly studied in order to arrive at the ætiological agent responsible for the disease. One of us (Radhakrishna Rao, 1933) showed from an intensive investigation of cases of cirrhosis of the liver in the King George Hospital, Vizagapatam, that malaria 'cannot be considered to be a causal factor in the production of cirrhosis of the liver, though it may be an important predisposing cause'. It is



important to remember that malaria and cirrhosis of the liver may coexist in the same patient without the former exerting any influence on the latter condition.

Finally, it may be said that the evidence presented in this paper proves that malaria *per se* is not a direct cause of cirrhosis of the liver.

#### SUMMARY.

In a previous communication (Tirumurti and Radhakrishna Rao, *loc. cit.*) it was pointed out that the common association of malaria and cirrhosis of the liver in India has given rise to divergence of opinion regarding the rôle of the former in the causation of the latter. A critical investigation of the morbid histological changes in chronic malarial livers by the application of different staining methods including the silver impregnation of the reticulum has confirmed our previous view that malaria *per se* is not a direct cause of cirrhosis of the liver.

#### ACKNOWLEDGMENTS.

Our thanks are due to Dr. P. Ramachandra Rao, M.B., B.S., PH.D. (Lond.), for help during the course of the investigation, and to Dr. A. Vasudevan and Dr. T. Bhaskara Menon for lending material for investigation.

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## CARCINOMA OF THE THYMUS.

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SEVERAL cases of tumour of the thymus have been reported within recent years. Much confusion still exists as to their origin and true nature and this is chiefly due to the fact that there is no general agreement concerning the origin and nature of the thymus. Recently, we had to report on a thymus tumour which was removed at the General Hospital, Madras, and this case forms the basis for this paper and affords an opportunity for discussing the malignant tumours of this gland. The subject of the growth was a Hindu boy of thirteen years of age and the tumour formed a mass situated in the lower part of the neck extending on either side to the posterior margin of the sternomastoid muscle. This was removed at operation, which was unfortunately fatal, and has been examined in detail.

### HISTOLOGICAL APPEARANCES (Plate IX, figs. 1 to 4).

Under the low power of the microscope there could be made out at the periphery of some of the sections a capsule of loose connective tissue fibres containing a few cells. The tumour appeared as a solid mass of cells, closely packed together. At one part, the section showed thyroid tissue in contact with the tumour cells (Fig. 1). A definite fine reticulum was seen in sections stained with Foot and Mênard's (1927) silver carbonate impregnation method, and the reticular fibres were intimately associated with the cells (Fig. 2). In places where the reticulum was particularly dense it appeared to divide the growth into small irregular areas. Under high power the cells showed a tendency to alveolar arrangement. According to their histological characters the cells could be divided into several types. The type of cells which predominated were small in size measuring  $5\mu$  with nuclei of  $3\mu$  diameter

### EXPLANATION OF PLATE IX.

- Fig. 1. Photomicrograph of a section of the growth with low magnification, showing a diffuse mass of closely packed cells. At one place thyroid tissue is seen in contact with the proliferating cells.
- „ 2. Photomicrograph of a section of the growth stained with Foot and Mènard's silver carbonate impregnation method to show the reticular fibres. Note their intimate relationship with the neoplastic cells.
- „ 3. Photomicrograph of a section of the tumour under high power showing the characters of cells. Note the small size of the cells with dense nuclei and reticulated cytoplasm. The polyhedral outline of some of the cells can be made out. Four larger somewhat oval cells with vesicular nuclei are also seen in this section. A mononucleated giant cell is seen in the centre of the field.
- „ 4. Shows an abortive Hassall's corpuscle.



Fig. 1.

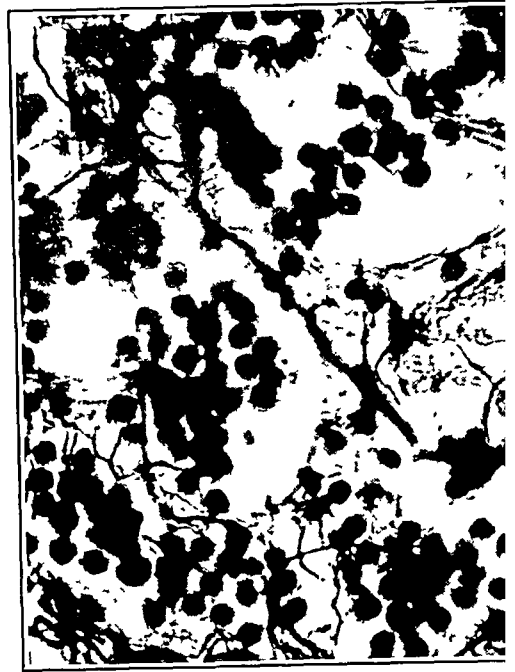


Fig. 2.



Fig. 3.



Fig. 4.



(Fig. 3). The cytoplasm was as a rule moderate in amount and acidophilic in reaction and generally deeply stained, but in some of the cells the cytoplasm was abundant and the nucleus was situated eccentrically, and in others it was only present as a narrow rim round the nucleus. The cells, as a rule, were slightly separated from one another and not mutually compressed. Their outline was polyhedral and the cytoplasm appeared to throw off protoplasmic processes which occasionally continued into fibrils. The epithelial structure of the cells was clearly seen under the one-twelfth oil immersion lens. The nucleus was usually compact, round and hyperchromatic. Mitotic figures were not observed. The mass of the tumour was made of these cells, but scattered among them were a few larger cells the cytoplasm of which was slightly acidophilic and small in amount compared to the size of the nucleus. These cells varied in size, were not polyhedral in shape but oval and irregular. The nuclei of these were oval in shape and usually vesicular and larger and less deeply stained than those of the smaller cells mentioned above (Fig. 3). The chromatin was irregularly distributed and collected in clumps. These cells would appear to correspond to the reticular cells of mesenchymal origin.

Occasionally one could see a giant cell with or without masses of chromatin, representing remnants of ingested cells.

Scattered all through the sections were small, spheroidal groups of concentrically arranged cells with large, somewhat hyperchromatic nuclei. These cells occurred in groups of three or four, or in larger aggregations. They were rather denser than the other elements of the tumour, tended to show the appearance of early keratinization, particularly about their periphery, and all the characteristics of young thymic corpuscles in an early stage of development. Many of them appeared to lie in free spaces in the alveoli of smaller tumour cells (Fig. 4).

The tumour cells showed no tendency to undergo degenerative changes. Necrosis was not observed. There were no plasma cells nor eosinophile cells.

The stroma was made up of thin strands of connective tissue and there were well developed blood vessels.

*Histological diagnosis.*—That the tumour has originated from the thymus is shown by the presence of Hassall's corpuscles though these are of a rudimentary nature. The polyhedral outline of the neoplastic cells and their tendency to form alveoli point to the epithelial nature of the tumour. The presence of abundant argentophil reticular fibres which are intimately associated with the neoplastic cells is indicative of their reticular nature. It is thus clear that this case belongs to the group of carcinomas derived from the epithelial reticular cells of the thymus. A strange fact noted in this tumour is the presence of dense deeply staining nuclei in which no structure could be made out. In this respect the present case differs from those already reported in which the nuclei of the tumour cells have been described as more or less vesicular with chromatin network and one or more nucleoli. The small size of cells is another noteworthy feature. The absence of karyokinetic figures and of any variation in the shape and size of the cells suggests that the tumour was of a relatively low grade of malignancy though it is not possible to say if there were any secondary deposits in the other organs as no post-mortem examination was made.

## COMMENT.

*Origin and nature of the cells of the thymus.*

As mentioned above, it is extremely difficult to classify tumours of the thymus. In order fully to comprehend the nature of these tumours, it is necessary to consider some points in the histology of this organ. The thymus is originally an epithelial structure, being an out-growth of the third branchial cleft on each side of the medial line, and consisting of several layers of cylindrical epithelium. At an early stage of development, this epithelial cell mass is invaded by lymphocytes.

The cortical thymus consists of densely packed masses of small round cells. These are called thymocytes and are morphologically identical with lymphocytes of the lymphoid tissue. Scattered between these cells are found elongated reticular cells with pale round or oval nuclei. The medulla consists of reticular cells similar to those of the cortex. It also contains Hassall's corpuscles—the characteristic structures of the thymus. The lymphocytes are much less numerous here than in the cortex.

It seems to be agreed that the Hassall's bodies and the vast majority of reticular cells are of entodermal origin. Hassall's corpuscles are formed from hypertrophic or degenerating reticular cells. A few reticular cells of mesenchymal origin are found in the vicinity of blood and in the capsule. The reticular epithelial cells become flattened from infiltration with lymphocytes and cannot be easily distinguished from the nuclei of the connective tissue reticular cells but the former do not take up any of the dye-stuff in vitally stained animals in contrast to the reticular cells of mesoblastic origin.

There is, however, considerable dispute as to the origin and nature of the small round cells of the thymus. Are these lymphocytes differentiated entodermal cells or are they derived from mesodermal tissue? Some histologists, Schridde (quoted by Kaufmann, 1929), for example, consider that the thymus is purely an epithelial organ and that the thymocytes or lymphocytes are of entodermal origin and that a true thymus tumour has an epithelial structure. According to this view a new growth of this region composed of lymphoid cells is a lymphosarcoma which has its origin in the lymphatic glands of the anterior mediastinum and is not of thymic origin at all. This view appears to have lost much ground since Maximow (1931) showed that the small round cells of the thymus were of mesodermal origin. Not only are they morphologically identical with the lymphocytes but they are equally susceptible to X-ray injury and are identical in their serological reactions. They both show the same type of amoeboid movement. Furthermore, the thymocytes are liable to be transformed into plasma cells and eosinophile myelocytes as are the lymphocytes. According to him these cells migrate into the thymus from outside and invade the primitive epithelial cell mass and multiply there. In this way, the original epithelial sheet is converted into a reticular cell mass. Many of these lymphocytes, no doubt, arise from the perivascular mesenchymal cells of the blood vessels of the thymus. This view may be said to hold the ground at present and we have reasons to oppose it. This preliminary statement of the origin and nature of the various cell types composing the thymus, we hope, makes the ground clear for a discussion of the various points of origin of the malignant tumours of the thymus.

### *Histogenesis of the thymus tumours.*

Tumours of the thymus may arise from the following types of cells:—

- (A) Reticular cells of entodermal origin of the medulla and cortex.
- (B) Hassall's corpuscles which are also of entodermal origin.
- (C) Lymphocytes of mesenchymal origin.
- (D) Reticular cells of mesenchymal origin.
- (E) Interlobular connective tissue and the capsule.

We shall now consider the tumours arising from these structures in greater detail.

*A and B. Reticular cells of entodermal origin and Hassall's corpuscles.*—Both these types of cell structures are epithelial in origin and the tumours derived from them would be called carcinomata. Several of these have been described: new growths resembling epitheliomas containing more or less developed Hassall's bodies or epithelial pearls, and tumours composed of epithelial cells suggesting an origin from the entodermal reticular cells of the thymus with or without rudimentary Hassall's bodies, and typical adenocarcinomas. Recently, Schuster (1927) has described a mucus-secreting glandular carcinoma of the thymus. The vast majority of these epithelial tumours appear to have originated from the reticular cells. There is no authentic record of a carcinoma of the thymus arising from Hassall's bodies and consisting of these structures alone. Multinucleated giant cells are usually seen in these tumours and they are probably formed by the rapid division of the nuclei of the reticular cells without a corresponding division of the cytoplasm.

*C. Lymphocytes or thymocytes.*—The tumours which originate from these elements are practically indistinguishable from the lymphosarcomata of the lymphoid tissues. These are composed of uniformly small round cells and are devoid of reticular fibres and Hassall's corpuscles. It appears, however, doubtful if a case of pure lymphocytoma of the thymus has ever been described, but leukæmic infiltrations do occur which may be mistaken for neoplasms of this type. In some of these cases reversion of the lymphocytes to the primitive type cell may be so pronounced that the histological features resemble very closely those of a large round cell sarcoma. Simmonds (quoted by Kaufmann, *loc. cit.*) suggests the name 'thymoma' for tumours arising from these cells. This view is of course based on the assumption that the thymocytes are peculiar to this gland and are of epithelial origin. But as these cells are now regarded by many workers as derivatives of mesoblastic elements which wander into the primitive epithelium of the thymus during its early development, this designation is unsatisfactory. A much better term is 'lymphocytoma' and it is better to avoid the term 'thymoma'. On the contrary, Jaffé (1926) is of the opinion that the term 'thymoma' should be restricted to tumours arising from the epithelial elements of the thymus gland containing Hassall's bodies. We share this view though we do not attach much importance to the presence of Hassall's bodies. These structures, it appears from a study of the literature, are not always present. If the term 'thymoma' is to be used at all, it should denote a tumour of the thymus of undoubted epithelial origin, i.e., arising from those cells which constitute the essential elements of this organ. It cannot be used to designate a tumour which has its origin in lymphocytes of mesoblastic origin which really do not belong to the organ but only migrate into it from outside and are neither characteristic nor peculiar to the thymus tissue.



*D. Reticular cells of mesenchymal origin.*—A tumour arising from these cells may be termed lymphosarcoma or large round cell sarcoma. The most suitable name, however, is reticular cell sarcoma. Most of the cases reported in the journals as lymphosarcoma of the thymus appear to have been derived from these cells. It may be noted that tumours arising from the reticular cells of entodermal origin show epithelial characters, while tumours of reticular cells of mesenchymal origin exhibit mesoblastic features, namely, irregularity of cellular outline, tendency to the formation of spindle cells, etc.

*E. Interlobular connective tissue and the capsule.*—A tumour arising from these tissues has the characters of a spindle-cell sarcoma. Two such cases have been reported by Simmonds (quoted by Kaufmann, *loc. cit.*). According to Ewing (1928) the origin of these tumours has never been completely traced to the connective tissue stroma and he is inclined to the view that the so-called spindle-cell sarcomas are really varieties of thymoma, i.e., tumours derived from the lymphocytes or the reticular mesenchymal cells of the thymus. Foot (1926) thinks that no such tumour has ever been described and he sounds a note of warning against regarding tumours composed of cells resembling large spindle cells as fibrosarcomas inasmuch as these cells may have been derived from entodermal reticular cells and are therefore of epithelial origin. In doubtful cases, the presence of Hassall's corpuscles even though of a rudimentary nature may indicate an entodermal origin.

#### *Relative incidence of malignant tumours of the thymus.*

In studying the reports of tumours of the thymus since 1926, we were greatly impressed with the fact that almost all the reported cases were of the carcinomatous type. We mention this because statements are frequently made in textbooks and journals that sarcomata are the commonest type of tumours found in the thymus. Rubaschow (quoted by Foot, *loc. cit.*) compiled a list of thymus tumours in 1911 and found 52 sarcomas, 12 carcinomas and 11 miscellaneous tumours. Excluding the miscellaneous tumours there were reports of 81 cases of thymus tumours up to 1926. According to Symmers and Vance (1921) only four cases of primary carcinomata had been reported up to the time they published their paper. Bringing the figures up to date, Foot stated in 1926 that his case was the seventh. Brannan (1926) reviewed the literature up to 1926 and found 15 authentic cases of carcinoma of the thymus. It will be noted that there is a considerable difference between the figures of Foot and Brannan but the fact remains that these authors and many others hold that the thymus carcinomata are of rare occurrence. It also emphasizes the extreme difficulty of determining the origin and nature of thymus tumours. Ewing says that lymphosarcoma is the most frequent form of thymus tumour. We have read the cases which have been reported or abstracted in the *Archives of Pathology* and other available journals since 1926 and have been able to collect 25 cases of tumours arising from the thymus region, which are as follows: Foot (1926), Brannan (1926), Brown (1926), Schuster (1927), Kaijser (1928—two cases), Meeker (1928), Matras and Priesel (1929—seven cases), Parabutschew (1930—four cases), Duguid and Kennedy (1930—two cases), Leyton, Turnbull and Bratton (1931—two cases), Craver (1931), Babès (1932), and McDonald (1932). We do not claim that the list is complete, but in our opinion it gives a fair idea of the incidence of malignant tumours of the thymus. From a study of these, it appears that there

were only two cases diagnosed as lymphosarcoma of the thymus during the period under review, but both of these had their origin in the mediastinal lymph nodes (Brown's case and Duguid and Kennedy's second case). There were, however, a few cases of mixed type in which both epithelial and lymphoid elements were present. We refer to the cases of Matras and Priesel and of Babès (quoted by McDonald, 1932), who classify thymus tumours on the presence or otherwise of lymphocytes and their arrangement. Such attempts have added to the confusion of an already difficult and complex subject. It appears doubtful whether the presence of lymphocytes in an epithelial tumour of an organ such as the thymus which is normally rich in these cells is of much significance. Moreover, as the growth of a reticular cell carcinoma proceeds the lymphocytes naturally tend to decrease in number and ultimately may disappear altogether, so that at different times and in different sections the proportion of the lymphocytes to the tumour cells (epithelial reticular cells) may vary considerably. Meeker's case also presents some difficulty as to its origin but according to the author the tumour cells showed a more or less close resemblance to the small thymus cells and the thymus reticular elements. Excluding cases of doubtful origin and nature there were 19 cases of primary carcinoma of the thymus. We have not been able to find a single case of undoubted primary lymphosarcoma of the thymus during the period under review. It is thus evident that since 1926 the tendency to diagnose carcinoma of the thymus has been very marked.

Is it not possible that some of the tumours diagnosed and reported as lymphosarcomas in the earlier literature represented an extension of the neoplastic process from lymph nodes of the anterior mediastinum to the thymus? A case of this type was recently described by Brown. It was a reticular cell lymphosarcoma of the thymus region, the origin of which was traced to the lymph nodes of the anterior mediastinum.

The possibility of a transformation of the Hodgkin's granuloma into a sarcomatous process has also to be kept in mind. That sarcomatous change may occur in the terminal stages of Hodgkin's disease is well known and cases of this type are not uncommon. Such a condition is called Hodgkin's sarcoma. It is conceivable that some of the cases described as lymphosarcoma of the thymus or thymoma in the literature are of this nature.

It may also be noted that Hodgkin's granuloma has been known to take on invasive characters. Shennan (1928) has described four tumours of the thymus composed of cells like those of lymphadenoma and he calls such tumours 'thymoma of lymphadenoma type'. They may either invade the surrounding tissues or may remain localized. There are, however, no convincing reasons for regarding these cases as lymphosarcomas of the thymus. In view of all these considerations we are inclined to believe that carcinomata of the thymus are not so rare compared with thymus sarcomata as is generally believed.

#### SUMMARY.

A case of carcinoma of the thymus arising from the epithelial reticular cells is described. It is composed of epithelial cells of small size showing a tendency to alveolar arrangement. Though well-developed Hassall's corpuscles were not found

A brief discussion on the origin and nature of malignant tumours of thymus is added. The suggestion is made to restrict the term 'thymoma' to tumours of the thymus which arise from the epithelial reticular cells.

The question of the relative incidence of thymus carcinoma and sarcoma is discussed with the conclusion that thymus carcinoma is not so rare compared with thymus lymphosarcoma as is generally believed.

BABES (1932)	..	..	Quoted by McDONALD (1932).
BRANNAN, D. (1926)	..	..	<i>Arch. Path.</i> , <b>1</b> , p. 569.
BROWN, S. E. (1926)	..	..	<i>Ibid.</i> , <b>2</b> , p. 822.
CRAVER, L. F. (1931)	..	..	<i>An. of Surgery</i> , <b>91</b> , p. 391.
DUGUID, J. B., and KENNEDY, A. M. (1930).			<i>Jour. Path. Bact.</i> , <b>33</b> , p. 93.
EWING, J. (1928)	..	..	'Neoplastic Diseases', 3rd ed.
FOOT, N. C. (1926)	..	..	<i>Amer. Jour. Path.</i> , <b>2</b> , p. 33.
FOOT, N. C., and MENARD, M. C. (1927)	..	..	<i>Arch. Path.</i> , <b>4</b> , p. 211.
JAFFE, R. H. (1926)	..	..	<i>Ibid.</i> , <b>2</b> , p. 627.
KAIJSER, R. (1928)	..	..	<i>Ibid.</i> , <b>5</b> , p. 733.
KAUFMANN, E. (1929)	..	..	'Pathology for Students and Practitioners', Translated by REIMANN.
LEYTON, O., TURNBULL, M., and BRATTON, A. B. (1931).			<i>Jour. Path. Bact.</i> , <b>24</b> , p. 635.
MATRAS, A., and PRIESEL, A. (1929)	..	..	<i>Arch. Path.</i> , <b>8</b> , p. 541.
MAXIMOW, A. (1931)	..	..	'A Textbook of Histology', 1st ed.
McDONALD, S. (1932)	..	..	<i>Jour. Path. Bact.</i> , <b>35</b> , p. 1.
MEKER, L. H. (1928)	..	..	<i>Arch. Path.</i> , <b>5</b> , p. 928.
PARABUTSCHEW, A. (1930)	..	..	<i>Ibid.</i> , <b>10</b> , p. 333.
SCHUSTER, H. (1927)	..	..	<i>Ibid.</i> , <b>3</b> , p. 142.
SHENNAN, T. (1928)	..	..	<i>Jour. Path. Bact.</i> , <b>31</b> , p. 365.
SYMMERS, D., and VANCE, B. M. (1921)	..	..	<i>Arch. Int. Med.</i> , <b>28</b> , p. 239.

## A DIET SURVEY OF SOME FAMILIES AND INSTITUTIONS IN CALCUTTA.

BY

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A COMPLETE diet survey in India is probably the most urgent and to-day should be the most fruitful field of investigation in the different lines of nutritional research. In the first place in few large areas of the world are there such a diversity in food habits due to social, economic and climatic conditions. Further in many cases certain types of disease are found in association either with certain areas or social groups. It is probably no exaggeration to say that there is a large and unexplored mass of material demanding a survey both dietetic and clinical which would be of value in assessing the precise rôle of nutrition in relation to disease. This expectation is enhanced by the impression the senior author (H. E. C. W.) has obtained from medical men in practice in India that malnutrition is, in their opinion, much more widespread and probably more complex than was hitherto realized. In the second place any improvement in the diet of the masses as a result of the survey must be a slow process and indeed rather in the nature of an expedient, hence if it is to be carried out without waste must depend on accurate quantitative and qualitative knowledge of the actual diet consumed and that actually or potentially available.

The present investigation was undertaken primarily as an initial step to correlate diet with physique and the incidence of clinical signs of disease. The field studies do not unfortunately include the poorer classes, nevertheless it can be taken that the diets recorded here are better and probably much better than what is consumed by the masses. It is hoped to obtain information on this class but for various reasons accurate data are difficult to procure. The present study includes ten middle-class Bengali Hindu families, a Bengali male hostel, two orphanages—one Muslim and one Hindu, an Anglo-Indian school, a Marwari gate-keeper and an Oriya sweeper

(the latter may be considered as representative of the poorer classes). The data on the families were collected by one of us (D. N. M.) who knew them personally and obtained figures concerning the food purchased and the age, number and sex of the members of each household. As regards the institutions, we had in each case the active co-operation of the managing staff who could supply us with the exact details of the food purchased and dispensed to the inmates each day. The latter data could be relied on as in every case the budget was limited and every attempt was made to make the most of the money available. The cost of the diet per head and per man value (henceforth M. V.\*) has been calculated at the current prices in Calcutta which it is hoped should serve as a guide as to what is possible for different classes of the community according to the income received. The income of the head of the household has also been given. This, unfortunately except as a rough index of their economic status, is not of much value in individual cases as the junior members of the household were often contributing their share in the food budget. The Bengali male hostel was probably a fairly good guide to the upper standard of living of unmarried wage earners of Rs. 40 per month with other obligations.

In this communication the composition of the diet has been analysed under the following heads: protein, animal protein, fat, animal fat, carbohydrate, ash, calcium, phosphorus, total calories, per cent of calories from dairy products and cereals respectively, and the per cent of the food budget spent on fruits and vegetable and dairy products. An analysis and discussion of the diets as regards vitamins will be made when the figures for the most important ones have been completed. In the Table are collected the data for each family, institution and individual. As the families are all Hindu and of the same social class they have been averaged and this figure compared with the other groups for comparison. The figures employed in the analysis of the diets were taken, as regards the major food-stuffs, for the most part from McCarrison's (1929) 'Food', while those for the minerals were derived from analysis carried out in this laboratory.

*Calories.*—The average calorie intake per M. V. ranges with two exceptions (one family and the gate-keeper) from 2,400 to 3,800 per day. In the case of the family with over 5,000 calories there was probably a certain amount of waste or leakage and in addition their food budget and income were high. The Marwari gate-keeper was an athlete (wrestler, stood 6' 3") and is hence not quite a typical sample. The data concerning the families are probably less accurate than the others owing to less careful management of the household. The data concerning the sweeper are probably most open to criticism but it is the general impression of the two senior authors (H. E. C. W. and B. A.) that it could scarcely be much better at least as regards quality. The calorie intake is in all cases up to that (2,400) recommended by the League of Nations (1935) as the minimum for a sedentary worker. In most cases, however, they fall short of that (3,400) recommended by the Committee of the British Medical Association (1933) and that (3,000) by the Joint Committee of the Ministry of Health (1934). It is expressly stated in the League of Nations' report that their figures refer to those living in a temperate climate and it would seem likely in view of the frequent necessity in India of planning diets for famine relief and possible future economic legislation that some standard should be sought. The figures for the orphanages are of interest in this respect.

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\* Lusk's co-efficients.

TABLE.

No.	Name.	MAJOR FOOD-STUFFS.					MINERALS.			CALORIC DISTRIBUTIONS.					MONETARY DISTRIBUTIONS.		
		Total protein, g.	Total fat, g.	Total carbohydrate, g.	Total animal protein, g.	Total animal fat, g.	Total ash, g.	Total phosphorus, g.	Total calcium, g.	On protein, per cent.	On fat, per cent.	On carbohydrates, per cent.	On cereals, per cent.	On dairy products, per cent.	Total cost in annas.	On vegetable and fruits, per cent.	On dairy products, per cent.
1	A. FAMILIES.																
	Bengali Hindu family. Approx. income Rs. 500. Heads 5.125 : M. V. 4.033.	4,663	187.46	592.46	40.51	90.61	21.90	2.98	1.54						11.06		
	Per day per head ..	5,924	238.17	752.74	62.90	115.13	27.82	3.78	1.96						14.05		28.12
2	Bengali Hindu family. Approx. income Rs. 500. Heads 14.0 : M. V. 11.85.	2,156	51.98	283.77	23.42	45.67	7.32	0.98	0.93						5.52		
	Per day per head ..	2,547	61.27	335.26	27.07	53.96	8.65	1.15	1.11						6.52		50.78
	Per day per M. V. ...																
3	Bengali Hindu family. Approx. income Rs. 500. Heads 15.0 : M. V. 12.69.	1,818	47.22	256.27	10.53	38.46	6.741	1.021	0.589						4.62		
	Per day per head ..	2,149	55.84	302.91	23.09	45.46	7.96	1.207	0.696						5.46		48.31
	Per day per M. V. ...																

\*M. V. = Mean value.

TABLE--contd.

[illegible]

7	Bengali Hindu family. Approx. income Rs. 200. Heads 60: M. V. 49.	3,145	73-97	63-44	549-3	6-69	17-25	12-18	1-257	0-423	9-04	18-7	71-6	62-20	5-58	5-40	44-7	12-33
	Per day per head	3,850	90-57	77-60	672-6	8-19	21-13	14-92	1-539	0-519						0-61		
	Per day per M. V.																	
8	Bengali Hindu family. Approx. income Rs. 60. Heads 70: M. V. 6-02.	2,793	54-07	83-81	437-04	12-76	10-88	7-905	1-025	0-313	7-03	27-00	64-15	50-00	7-81	4-19	28-30	15-60
	Per day per head ..	3,248	62-87	97-45	508-19	11-83	23-11	9-192	1-192	0-364						4-87		
	Per day per M. V.																	
9	Bengali Hindu family. Approx. income Rs. 60. Heads 110: M. V. 9-52.	2,913	64-30	75-66	474-50	20-66	21-69	8-953	1-159	0-383	9-05	24-19	66-79	56-27	6-87	4-26	40-26	23-2
	Per day per head ..	3,366	74-29	87-42	548-35	23-87	25-07	10-348	1-339	0-443						4-92		
	Per day per M. V.																	
10	Bengali Hindu family. Heads 90: M. V. 7-56.	2,912	67-11	93-50	474-70	23-27	20-6	7-96	1-225	0-679	9-78	21-00	69-21	66-57	7-44	3-13	16-83	31-01
	Per day per head ..	3,348	79-89	75-70	565-14	28-21	24-55	9-48	1-458	0-809						3-73		
	Per day per M. V.																	
	Average for families.																	
	Per day per head ..	2,871	67-83	84-54	440-62	22-84	34-51	10-40	1-365	0-664	9-55	26-96	63-48	54-48	12-99	5-66	29-21	28-43
	Per day per M. V. ..	3,411	80-59	100-67	523-19	27-03	40-96	12-39	1-622	0-793						6-67		

\*M. V. = Man value.



No.	Name.	Total calories.	MAJOR FOOD-STUFFS.				MINERALS.			CALORIC DISTRIBUTIONS.					MONETARY DISTRIBUTIONS.			
			Total protein, g.	Total fat, g.	Total carbohydrate, g.	Total animal protein, g.	Total animal fat, g.	Total ash, g.	Total phosphorus, g.	Total calcium, g.	On protein, per cent.	On fat, per cent.	On carbohydrates, per cent.	On cereals, per cent.	On dairy products, per cent.	Total cost in annas.	On vegetable and fruits, per cent.	On dairy products, per cent.
11	B. INSTITUTIONS.																	
	Muslim Orphanage. Heads 202-16; M. V.* 164-00.																	
	Per day per head ..	2,203	72-96	31-75	392-37	8-62	3-12	10-16	1-40	0-40								
	Per day per M. V. ..	2,702	89-49	38-94	431-25	10-58	3-33	12-47	1-72	0-49								
12	Hindu Orphanage. Heads 20-8; M. V. 20-8.																	
	Per day per head																	
	Per day per M. V.	2,783	65-10	42-64	517-07	10-72	10-85	7-96	1-25	0-25								
13	Bengali Male Hostel. Income Rs. 40 per head. Heads 16-0; M. V. 16-0.																	
	Per day per head																	
	Per day per M. V.	2,499	51-17	51-97	440-4	13-65	12-47	6-55	0-001	0-25								

[illegible]

\* M. V. = Mean value.

As mentioned above the data were presented us from entirely independent sources and without any bias on our part except that of emphasizing the importance of accuracy. The boys appeared active and free from disease and in all cases their appetites were satisfied. They were also weighed regularly by the authorities. Detailed data as regards height, weight, physique by the A. C. H. (arm, chest, hip) index of nutrition are being collected and will be published later. It will be noted that the calorie intake per M. V. all fell remarkably close at 2,800 which is all the more interesting in that, as regards the Hindu orphanage, the M. V. and per head value were identical, the boys being all over 14 years of age. The proposal to accept the calorie intake of active boys who are subjectively satisfied as regards quantity would be a not unreasonable figure to use as a safe or even maximum figure for men doing light work in this country.

*Protein.*—The protein element in nutrition in India may ultimately prove to be the most important and it certainly will be the most difficult to remedy. At the outset of this discussion a quotation of a few of the more recent Western standards should not be out of place. The British Medical Association Committee recommended 100 g. of protein of which 50 g. should be of good quality (animal sources). This would constitute about 12 per cent of the total calories of their diet. The Ministry of Health Committee modified the animal protein to 37 g. and accepted a total protein intake of 100 g. This would constitute 13 per cent of the total calories. As regards children the protein requirements are somewhat greater relatively than adults and should hence constitute a greater percentage of the total calorie intake.

As regards the protein intake of the families this is found to vary from 39 g. to 115 g. per day per M. V. with an average of 80 g. of which 27 g. are from animal sources. As the average calorie intake is relatively high the protein calories constitute only about 9 per cent of the total, a figure which falls below those recommended above, namely 12 per cent to 15 per cent. It should be pointed out, however, that any waste is much more likely to affect the cheaper foods such as rice which is poor in protein. This would tend to increase the percentage of calories derived from this source without decreasing the total to any considerable extent. The institutions are somewhat above or below what is consumed by the families as regards the protein intake, while the animal protein is exceptionally low, 9 g. to 13 g. per M. V. in the two Indian institutions and 29 g. per M. V. in the Anglo-Indian school. The percentage of calories derived from protein is not above and in the case of the Hindu orphanage rather below the standard. As these are children's institutions it will be appreciated that the protein intake is probably definitely below optimum in view of the fact that the relative requirements of young people for this food-stuff are greater than for adults. As regards the Marwari gate-keeper who is hardly a true representative sample his total protein intake is 144 g. of which, however, only 5.2 g. come from animal sources. His staple cereal is wheat with dhal and only occasionally was rice taken. The Oriya sweeper is probably fairly typical of his class. He is living on 44 g. of protein a day which is about the lower level recommended by Chittenden. This protein is for practical purposes almost entirely derived from cereals, such as rice, which in addition is relatively poorly absorbed. It is difficult to see how real health could be maintained over a long period on such a diet. The male adult hostel presents a poor picture and is probably fairly representative of the diet of a class earning not more than Rs. 40 per month. It falls

short of the Western standards in almost every respect and it may be assumed that the diet of many other such wage earners can be little better than this.

*Fat.*—The figures for fat when compared with Western standards with the exception of the families are distinctly poorer than in the case of protein. The accepted standard is about 100 g. per day of which 50 g. is animal fat. All the institutions have a fat intake of not more than 50 g. of which in the case of the two orphanages only 3 g. to 12 g. is from animal sources. The Anglo-Indian school presents an apparently better standard with 34·6 g. of animal fat which, however, was derived almost entirely from suet or lard. It is probable that the fat requirements in the tropics are less than what is recommended in Europe but it would seem unlikely that health could be maintained on such a low proportion of animal fat. An adequate fat intake has yet to be found for tropical climates.

*Minerals.*—The two minerals investigated, namely calcium and phosphorus, would when compared to that recommended by Sherman appear to be distinctly below what is adequate, especially for children. Fortunately, the low calcium is associated with a correspondingly low phosphorus intake which probably means a more effective absorption of both than might otherwise be the case were one or other in excess. Sherman recommends about 1 g. calcium per day for a child, while the intake in the institutions varies from 0·23 g. to 0·40 g. per head. In the case of the Anglo-Indian school (0·2 g.) the Headmaster reported that some of the boys had been seen eating the plaster off the walls which is significant although this might be interpreted on other lines than natural instinct or craving. The calcium intake of the families is, however, quite within the accepted standard and it should be mentioned that adult Indians may obtain a certain amount of calcium from chewing betel leaves containing a lime paste flavoured with catechu. It is possible, however, in view of the ample sunlight and the even balance of calcium and phosphorus in the diet that the minimum requirements may be less than that recommended by Sherman. Again, however, it is difficult to conceive that it should be as low as 0·20 g. per day which in the case of the children is derived from sources other than milk. The higher calcium intake of the Muslim orphanage is due to the high consumption of atta (whole-wheat), while the low value in the Hindu one is due to the preponderance of rice in the diet. The low figure for the Anglo-Indian school was due to the fact that they consumed white bread and rice, both of which are poor in this mineral.

*Distribution of calories.*—The percentage distribution of calories between protein, fat and carbohydrate used to be and still is to a great extent a good guide as to the general balance of a diet. Within recent years, however, owing to the increasing significance and importance for good or ill of such foods as milk products, cereals, etc., analysis has taken on a less chemical aspect and tends to assess values in terms of food-stuffs belonging to a general class. Such a development is to be encouraged particularly when it is borne in mind that the terms protein, fat and carbohydrate, although accurate and valid from a chemical point of view, may have less significance in the total economy (metabolism in the wide sense) of the organism which is primarily concerned in the handling of food-stuffs or food as a whole. This is the more important in view of the fact that many cereals, although chemically almost identical, often appear to be associated with particular pathological conditions, such as maize with pellagra, rice with beri-beri, wheat with defects in the nervous system (Mellanby, 1934), cabbage with goitre—conditions which in the

human subject have not yet been exhaustively explained in terms of deficiency of vitamins alone.

In this investigation the distribution of the diet has been calculated in terms of the percentage distribution of calories between cereals and milk products respectively and the percentage of the food budget spent on milk products and vegetables. This monetary basis has been adopted with the latter in view of the fact that valuable vegetables in a diet may contribute but little to its calorie value. The full significance of vegetables will be much more easily assessed when figures for the vitamin content of the food-stuffs have been worked out.

In the U. S. A., Rose (quoted by Sherman, 1932) recommends a percentage distribution of calories from cereals and milk products of 24 per cent to 37 per cent and 22 per cent to 32 per cent respectively for children. For adults somewhat less milk and more cereal is accepted as a suitable figure for an adequate diet. The examination of the figures shows that they fall short considerably from those mentioned above, particularly in the case of the institutions. The maximum for dairy products is 3·57 per cent in the Anglo-Indian school and this fraction costs 11 per cent of the food budget.

On the other hand the cereal calories are high amounting to 82 per cent of the total in the Muslim orphanage. The percentage of the budget spent on fruits and vegetables in this institution is 9 per cent to 17 per cent, a reasonable figure, although at certain times of the year this will represent vegetables which require cooking as the cheaper fruits such as mangoes and oranges are not available. As regards the families the percentage of calories derived from dairy products ranges between 2·4 per cent and 29 per cent, average 12·99. In many cases the milk products have been the means whereby a rather mediocre diet has been largely improved. The percentage of calories derived from cereals on the other hand, although it varies widely, is on the average 54 per cent—less than that obtaining in the children's institutions but higher than Sherman's figures. The percentages of the calories derived from these two sources show up very clearly the way in which the Indian diets recorded here differ from those generally advocated in the West.

The solution of the problem of malnutrition in India in all probability will converge round a qualitative and quantitative balance of these two sources of food.

*Cost of diet.*—The cost of the family diets with two exceptions are extraordinarily low, ranging between annas 2·89 and 6·52 per M. V. per day. The cheapest diet of all was that of the sweeper, annas 2·21 per day. The average for all the families is only annas 6·65 per M. V. per day and taking everything into consideration they are by no means too bad. The figure, however, cannot be taken as the minimum cost of such a diet. In point of fact it might be improved with a reduction in cost particularly if carried out on a larger scale. The cost, however, in the institutions and in particular the hostel merits special attention. The average income of the men in the hostel is Rs. 40 per month. In general it may be assumed that each member is independent and has to feed and clothe himself and possibly remit money home. The standard of living is probably as good or even better than many students in Calcutta can afford to live at. The diet is possibly just adequate for a sedentary worker if the standard figure suggested by the League of Nations is

accepted. The other constituents speak for themselves and the cost is annas 4·13 per M. V. per day. The diet is qualitatively very little better than the two Indian orphanages which cost about annas 2·6 to 2·9 per M. V. per day, while it is distinctly poorer than that of the Anglo-Indian school which amounts to annas 4·13 per M. V. per day.

The poorest diet all over is that of the Oriya sweeper, the calories derived from milk products are less than 2 per cent of the total and cost 10 per cent of his budget. Taking the diets all over the best value for the money would appear to be that of the orphanages and school. The cost of the Anglo-Indian school is annas 4·13 per M. V. per day, almost 33 per cent more than the other two. As far as India is concerned a diet such as this school provides could be procured at a slightly lesser cost by substituting atta for white flour which is cheap and incidentally would increase the Ca and P content. In addition this school used small quantities of European manufactured products such as cocoa and jam which might be replaced by some local sweatmeats at a lower cost. An average cost of about annas 4 per M. V. per day is a likely figure for such a diet, which apart from theoretical reasons, appears to work reasonably well as regards the health and growth of the children. In all these institutions, however, the diets might be passed taking Western standards into consideration, with a reasonable assurance of adequacy if one pint of milk per head were added. This would increase at the cost by about annas 1·5 per day bringing the total figure per M. V. per day for the diet to between annas 4·4 and 5·6. As far as the inhabitants of Calcutta are concerned and with present prices this would entail an expenditure of Rs. 7 to Rs. 10 per month per head which is undoubtedly out of the question for the masses and even for a large number of wage earners of Rs. 40 to Rs. 80 per month with families to keep.

#### DISCUSSION.

There can scarcely be any question that all the diets analysed fall below Western standard in most respects although in varying degree. In short they are all-round deficient diets. At the same time analysis shows this deficiency to have a bias in certain directions, namely animal protein, animal fat, dairy products, calcium and excess of cereal. Unfortunately these deficiencies are more marked in the children's institutions and the same is likely to hold good for children of parents who would belong to the class living in the above-mentioned hostel. Assuming that the Western standard is not rigidly applicable in India and this is probable in the case of fat, the degree of divergence between the figures collected here and the accepted standard is too great to be dismissed as falling within the range of what constitutes a good diet or what the human species can adapt itself to. It would appear to be justifiable to recommend a reduction in the quantity of cereal, an increase in the proportion of atta and an increase of milk products. At the present time the simplest expedient would be an increase in the consumption of milk or milk products. In a population where meat is largely forbidden and fish not everywhere obtainable, milk, which is permitted by nearly all, would supplement most of the deficiencies, covering in addition those properties present in atta the introduction of which into rice-eating areas though relatively cheap would be by no means easy.

The relation between deficient diet and disease is yet to be worked out in India. It appears to the writers that more accurate data on food consumptions and detailed

analysis of disease covering infant and maternal mortality and morbidity, diseases of children and certain conditions of middle age will have to be collected before any real correlation can be made.

It is unlikely that any one deficiency, such as a vitamin or mineral, will be the cause of any condition. The peculiar qualities of certain different types of food, chemically similar, such as maize or atta, may, with or without a vitamin deficiency, appear to condition certain departure from health. The possibility of certain deleterious properties of foods, such as the toxamins, should not, however, be sufficient reason to discard them for consumption, other factors being taken into consideration.

#### CONCLUSIONS.

(1) The diets analysed in this survey are poor in total and animal protein, total and animal fat, calcium and to a lesser extent phosphorus. They contain too low a percentage of dairy products and an excess of cereal.

(2) The minimum cost in Calcutta at current prices to-day of a diet which approaches to within a reasonable degree the Western standards is somewhere in the region of annas 4.4 to 5.6 per man value per day. Under present conditions this is beyond the means of most.

(3) As an expedient to improve the diets an increase in the consumption of milk products would be the easiest to introduce and in the long run the cheapest.

(4) An increase in the consumption of atta in the rice-eating districts is recommended.

(5) The collection of more data on food consumption in India is strongly urged in order to obtain suitable dietary standards.

(6) The association of diet with disease in India can only be determined by a careful diet survey in relation to clinical observations within the same area.

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## THE BASAL METABOLISM OF YOUNG MEN AT HYDERABAD (DECCAN) WITH A STUDY OF THEIR PHYSICAL CHARACTERS\*.

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### INTRODUCTION.

EVER since the remarkable piece of work by A. Ozorio de Almeida (1921) in Brazil, the interest of the workers in metabolism has been aroused as to the influence of the racial and the climatic factors in modifying basal metabolism. de Almeida, who studied the basal metabolism of 10 white men and 10 negro labourers using a Tissot gasometer, discovered that the metabolism of the whites was considerably lower than the generally accepted standards for the United States and that the metabolism of the negroes, though higher than that of the whites, was still much below the figures obtained in the temperate zones.

These findings of de Almeida have not been substantiated by many other workers. Prior to de Almeida's work, Eijkmann (1896, 1921), using a Zuntz-Geppert apparatus, studied 12 Malays and 11 Europeans living in Batavia and found no difference in the metabolism of the Malays, Europeans in Batavia and Europeans studied in Germany by Geppert, Loewy and Magnus Levy. Takahira (1925) studied the basal metabolism of 120 Japanese men and women and came to the conclusion that there was no significant difference in metabolism between the Japanese and the Europeans.

On the other hand Montoro (1921, 1922) in Havana obtained results similar to those of de Almeida. The results obtained by Knipping (1923) and confirmed by Fleming (1925) show that prolonged residence in the tropics tends to lower the basal metabolism.

Though the evidence is in favour of the inference that residence in the tropics tends to cause lowering of the basal metabolism, recent investigations suggest that

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there is also a racial factor involved. MacLeod, Crofts and Benedict (1925) studied 7 Chinese and 2 Japanese women who had been students in American colleges for at least fifteen months under the same climatic, dietetic and other conditions as Western women. Their metabolism averaged 10·4 per cent below the Harris Benedict standards (1919) for women and 10·2 per cent below the Aub-DuBois standards (1917). The authors have found both these standards about 5 per cent too high for American college women. But even then this leaves the Oriental women about 5 per cent below the American standards. This is in spite of the fact that for over a year the environmental conditions were exactly the same in both the cases. Williams and Benedict (1928) and Shattuck and Benedict (1931) carried out a series of studies on the Maya Indians in Yucatan in connection with the comprehensive programme of the Carnegie Institution of Washington for investigating racial metabolism. They used a field respiration apparatus. These investigations brought out the extraordinary fact that the average metabolism of the Mayas was somewhat above the basal metabolism of the North American whites. This is the first report in which a tropical or sub-tropical race has shown metabolism higher than that of the American or the European standards.

Again, in the third expedition to Yucatan, Steggerda and Benedict (1932) re-investigated the metabolism in the Maya Indians. They confirmed the results obtained previously that the male Mayas have a high basal metabolism and a low pulse rate. A group of 30 male Mayas were found to have heat production on the average 8 per cent above the prediction standards for the whites. They definitely concluded that this high metabolism was largely due to the racial factor involved. This will be discussed later along with the other factors which have been supposed to influence metabolism.

*Basal metabolism observations in India.*—In India very few observations have so far been made. Mukherjee (1926) reported the results of observation on 15 male Bengalis between the ages of 22 and 27 years. The measures were carried out with the Douglas bag and the Haldane's gas analysis apparatus when the subject had been without food for 16 to 18 hours at a room temperature averaging 27°C. The metabolism on the average was 9 per cent below the Sanborn standards (1933). In a second paper Mukherjee and Gupta (1931) reported a new series of healthy Bengali men of ages from 20 to 29 years whose metabolism averaged 13·3 per cent below the DuBois standards with individual variations of from -0·3 to -31·2 per cent. Sokhey (1927), using a Collin's chain compensated gasometer (a modification of Tissot's gasometer) with the Haldane's air analysis apparatus (Henderson's modification), measured the basal metabolism in 21 male medical students in Bombay between 19 and 30 years of age. He found that 15 of his subjects showed a basal metabolism 10 to 23 per cent lower than the DuBois standards. The details of the experiments are not given.

Recently, Mason and Benedict (1931) have investigated the basal metabolism of 54 women (Tamils, Malayalis, Telugus, Coorgs and Kanarese) in South India of ages ranging between 17 and 31 years by means of Benedict's portable apparatus. They obtained results 16·9 per cent lower than the Harris-Benedict standards and 17·2 per cent lower than the Aub-DuBois standards and conclude that there is a definite racial factor.

All these investigations were carried out at seaside places. So far as I am aware, with the exceptions of the tests done at Lucknow which are discussed elsewhere, basal metabolism investigations have not been reported from the interior of India. The present investigations were undertaken with a view to study the physical characters of young men in Hyderabad which is situated at a higher level (nearly 1,700 feet above sea-level) and has a drier climate than those places in India whence the basal metabolism results have so far been received.

### TECHNIQUE.

The apparatus used was the Sanborn Motor-Grafic Metabolism Tester, manufactured by the Sanborn Company, Cambridge, Mass. It is a spirometer type of respiration apparatus based on the same principle as the Benedict-Roth apparatus. It consists of an inverted bell supported by a counter-weight and floating on water. The bell is filled with medicinally pure oxygen from a small cylinder which is conveniently fixed to the trolley carrying the apparatus. The subject breathes into and out of the bell through a rubber mouthpiece, the nose having been closed by means of a nose-clip. The rubber mouthpiece is connected to an oxygen control valve. When this valve is open, the subject breathes the outside air; when it is closed, the subject is connected to the breathing circuit of the apparatus. The expired air is led through a vessel containing sansoline (supplied by the Sanborn Company) which absorbs the carbon dioxide and the water vapour and the subject breathes in almost pure oxygen. The movements of the bell which correspond to the respiratory movements of the subject are registered on a chart applied to a revolving cylinder which moves by means of clockwork.

The apparatus indicates only the oxygen consumption of the subject and in this respect the results are not so accurate as those obtained by the Douglas bag or the Tissot methods, which measure both the oxygen consumption and the carbon dioxide production of the body from which the actual respiratory quotient may be calculated. On the other hand, a great advantage of the apparatus is that the results are graphic and permanent (Beaumont and Dodds, 1935). The rate of the absorption of oxygen is indicated by the rate of decline in the level of the writing-ink pen which is attached to the spirometer. This method offers a means of checking up leaks that may develop in the apparatus during the test; a very rapid fall in the level of the pen being caused by a leak. Another great advantage of this method is that it registers the mode of breathing. It is reported by Earle and Goodall, and Hannon and Lyman (quoted by King, 1924) that only subjects in whom breathing is regular give satisfactory results by the close system, and other results are readily weeded out by the record of respirations upon a kymograph. This is certainly true to some extent. I have found that the most constant and accurate results are obtained when the breathing is uniform and regular. If the breathing is irregular, the results obtained are almost invariably too high.

Another advantage of the apparatus is the convenience in calculation; the oxygen consumption per minute is directly read off from the chart, thus eliminating the personal factor in the measurements of the gases.

The apparatus can be used with the motor blower or without it. In the latter case rubber valves are substituted for the motor blower. The motor blower was used, however, only in a few cases. It was found that, with the motor blower in

TABLE I.

Subject number.	Name.	Age, years.	Body-weight (without clothes), kg.	Height, cm.	Sitting height, cm.	Pelidisi.
1	A. Ahmed ..	24	51.7	174.7	90.6	89
2	A. Manan ..	20	58.5	171.2	87.3	96
3	S. A. Khan ..	23	50.8	170.7	88.9	90
4	L. A. Khan ..	23	56.2	170.7	88.9	93
5	S. A. Husain ..	23	47.2	167.9	88.1	88
6	M. Y. Zubairi ..	22	55.8	171.5	90.2	91
7	M. Imamuddin ..	21	45.4	166.8	87.6	88
8	T. N. Reddi ..	20	55.8	174.0	89.4	92
9	S. M. Ali ..	22	55.5	162.0	..	..
10	S. A. Husain ..	27	59.0	166.4	84.6	99
11	A. Mohiuddin ..	19	55.0	175.5	89.7	91
12	R. R. Saksena ..	22	48.5	168.6	85.8	92
13	H. A. Ansari ..	21	46.5	168.3	86.9	91
14	M. Sharfuddin ..	19	43.2	166.0	87.6	86
15	M. Musiquddin ..	20	45.4	168.6	87.9	87
16	Jagmohan ..	20	52.0	166.6	85.8	94
17	Bhushnam ..	21	49.0	160.2	86.4	91
18	I. Rahman ..	20	56.5	178.6	91.4	90
19	Q. S. A. Rashid ..	22	52.5	170.7	90.9	89
20	K. S. Manvikar ..	22	71.3	177.7	93.9	95
21	R. Karan ..	22	53.0	177.0	..	..
22	Q. Ali ..	20	55.5	174.0	90.0	91
23	M. R. Beg ..	20	63.0	174.5	94.2	98
24	I. Ahmed ..	21	57.0	164.0	86.6	96
25	R. N. Abhyankar ..	32	54.0	165.1	87.9	93
26	P. Shankarya ..	22	57.2	162.2	83.8	99
27	X. Ramiah ..	20	46.5	164.5	86.4	90
28	S. Ramiah ..	19	41.5	168.0	86.4	86
29	R. B. Phatkay ..	20	56.2	172.0	90.3	91
30	I. G. Gadray ..	28	60.2	159.5	85.3	99
31	Ranade ..	31	68.2	162.5	87.6	100
32	V. G. Gadray ..	23	68.2	167.0	89.1	99
AVERAGES ..		22	54.2	169.3	88.3	92 ± 4

*Sitting height.*—The sitting height was actually measured in every case with all the precautions emphasized by Dreyer and Hanson (1921). As mentioned by Pirquet (1922), the sitting height thus measured equals, in Westerners at least, one-half of the total height plus 5 cm. Table II shows the actual sitting heights obtained in the case of my subjects as compared with the sitting heights calculated according to the formula,  $h/2$  plus 5 cm. where  $h$  stands for the total height. It is seen that the actual sitting height in most of the cases is less than the calculated sitting height. The average sitting height works out as 1.2 cm. less than the calculated average. From this it is seen that the average young Indian has a stem height about 1.2 cm. less, and therefore correspondingly longer legs, than the average Westerner. Even greater difference in sitting heights was found by Mason and Benedict (1931) in the case of their women subjects. They found that with the Tamils one-half of the average total height was 77 cm. and the actually measured sitting height was, on the average, only a little over 1 cm. greater than this. Much the same picture was shown by their other groups.

TABLE II.

Subject No.	Height, em.	SITTING HEIGHT, CM.			BODY-WEIGHT (WITHOUT CLOTHES), LB.			DEVIATION FROM NORMAL.		
		Actual.	Calculated.	Difference.	Actual.	American standards.	Difference.	Harris-Benedict, per cent.	Aub. DuBois, per cent.	Sanborn, per cent.
1	174.7	90.6	92.3	1.7	114	146	32	14.5	17.1	—
2	171.2	87.3	90.6	3.3	129	136	7	6.7	7.9	—
3	170.7	88.9	90.3	1.4	112	137	25	2.7	0	+
4	170.7	88.9	90.3	1.4	124	138	14	5	6.8	—
5	167.9	88.1	88.9	0.8	104	134	30	1	1.2	—
6	172.7	90.2	91.3	1.1	123	140	17	2.7	0.4	+
7	166.8	87.6	88.2	0.6	100	120.5	20.5	17.4	19	+
8	174.0	89.4	92.0	2.6	123	140.0	17.0	10	11	—
9	162.0	87.0	86.0	1.0	122	125	3.0	9	16	—
10	166.4	84.6	88.2	3.6	130	134.5	4.5	14	10	—
11	175.5	89.7	92.7	3.0	119	140	21.0	0.8	4.3	—
12	168.6	85.8	89.3	3.5	107	134	27.0	6	7.8	—
13	168.3	86.9	89.1	2.2	102	131	29.0	16	17.7	—
14	166.0	87.6	88.0	0.4	95.5	136	40.5	8	2.3	+
15	168.6	87.9	89.3	1.4	100	132	32.0	1	4.5	+
16	166.6	85.8	88.3	2.5	114	128	14.0	3	2.2	+
17	160.2	86.4	85.1	1.3	108	119.5	11.5	11	12.5	—
18	178.6	91.4	94.3	2.9	124	148.5	24.5	8	8.8	—
19	170.7	90.9	90.3	0.6	115.5	136	20.5	1	4.15	—
20	177.7	93.9	93.8	0.1	157	148	9.0	10.5	10.5	—
21	177.0	87.0	93.5	6.5	117	144	27.0	11.5	14.65	—
22	174.0	90.0	92.0	2.0	122.5	140	17.5	9	10	—
23	174.5	94.2	92.2	2.0	139	142	3.0	8	7.6	—
24	164.0	86.6	87.0	0.4	125	125	0	17	17.2	—
25	165.1	87.9	87.5	0.4	119	135	16.0	10	16.4	—
26	162.2	83.8	86.1	2.3	125.4	125	0.4	4.25	5.15	—
27	164.5	86.4	87.2	0.8	102	126	20.0	0.6	1.5	—
28	168.0	86.4	89.0	2.6	91	130	39.0	11	14.6	+
29	172.0	90.3	91.0	0.7	123	137	14.0	12	13.4	—
30	159.5	85.3	84.7	0.6	132	125	7.0	9.2	11.9	—
31	162.5	87.6	86.2	1.4	150	130	20.0	6.5	7.8	—
32	167.0	89.1	88.5	0.6	150	133	17.0	6	4.9	—
AVERAGES	..	..	..	1.2	119.3	134.3	15.0	6.8	8.7	7.4

*Pelidisi*.—The usual clinical classification of well-nourished, moderately well-nourished, poorly nourished, etc., is not precise and therefore not satisfactory as an indication of the state of nutrition of an individual. As the basal metabolism is affected by the nutritional state of the individual, it is essential for the study of the normal subject to have a precise knowledge of his state of nutrition. The Nutrition Laboratory of the Carnegie Institution of Washington has found the pelidisi of Pirquet of great advantage over the ordinary, rather gross, clinical indications (Shattuck and Benedict, *loc. cit.*).

Pelidisi is the relationship between the weight of the individual and his sitting height. This method of fixing the state of nutrition of an individual was first originated by the late Professor Clemens Pirquet (*loc. cit.*) of Vienna and is known as the Pirquet index.

It is expressed by the formula

$$\sqrt[3]{\frac{10 \times \text{weight in g.}}{\text{Sitting height in cm.}}}$$

and the result is multiplied by 100.

In the original preparation of the material, Pirquet assumed that for small children a pelidisi of 100 represented the ideal. It has since been found that for adults a value somewhat less than this, probably nearer 97 or 98, represents the normal state of nutrition. It has been found that with Westerners a pelidisi around 90 represents a distinctly low state of nutrition and that a pelidisi over 100 represents a fat person.

Table I shows the pelidisi in the case of my subjects as it has been calculated from the sitting height actually obtained. The majority of the results are over 90, the minimum being 86 in the subjects Nos. 14 and 28, the maximum being 100 in the subject No. 31. The average for 30, out of the 32 subjects, works out as  $92 \pm 4$ .

It is, therefore, clear that the pelidisi as obtained in these subjects is less than that which is accepted as representing the normal state of nutrition in the Westerner. Does this denote that these subjects were under-nourished? I do not think so. Exceptions might be made in the case of subjects Nos. 14 and 28 who were unusually thin. The rest were certainly in good health and were getting good food. It would probably be more correct to infer from the results that a pelidisi of about 92 indicates the normal state of nutrition in young Indians. That the pelidisi showing the normal state of nutrition may differ in different races is suggested by the measurements in the Chinese. Stevenson (1928), after examining over three thousand of the Chinese, came to the conclusion that the Chinese approaches the pelidisi of 90 as the basic standard. Benedict and Meyer (1933) found that the average pelidisi of their 18 American-born Chinese girls (12 to 22 years of age) was 92.

*Blood pressure*.—The blood pressures were taken after the basal metabolism tests were over and before the subject had partaken of any food. The instrument employed was of Riva Rocci type fitted with mercury monometer, the zero point of which was carefully adjusted. The elastic cuff was about 12 cm. broad. This is assumed to give an error of from plus 7 to plus 9 per cent compared with the pressures obtained directly from the artery (Norris, Bazett and McMillan, 1928). The auscultatory method was employed. The subject was made to sit on a stool with the left hand, which was used for examination, resting relaxed on a table in front,

palm upwards. In this posture the brachial artery was level with the heart. Readings were taken during release of pressure. Readings of the systolic pressure was taken at the point at which the first clear thump was heard below the cuff (first phase), the stethoscope being snugly applied to the artery. The diastolic pressure was used at the so-called fourth phase, i.e., at the point where the second clear thump becomes suddenly muffled. Several consecutive readings were taken to ensure correctness of results, the pressure between the readings being allowed to fall to zero and sufficient time permitted to elapse for the venous pressure to fall to normal level. The psychical element of interference in the case of these subjects was negligible and did not interfere in obtaining correct results.

The blood-pressure estimates show that the readings are invariably lower than what has been taken as normal for Westerners. The average systolic pressure obtained was  $100.4 \pm 11$  mm. of mercury, the maximum being 125 mm. in subject No. 4 and the minimum being 75 mm. in subject No. 22 (Table III). The normal systolic pressure in Westerners as studied by Melvin and Murray (1914) was 112 mm. for young men between the ages of 20 and 29 years. Fisher (quoted by Norris *et al.*, *loc. cit.*) found that, in 64,574 cases studied by him, the average auscultatory systolic pressures was 123.2 mm. ranging between 116 mm. at 16 years and 136 mm. at 65 years. More important are the results obtained by Alvarez (1920, 1923) who, unlike Fisher, did not base his results on insurance statistics but studied students in a State-controlled University. He found that 45 per cent of the male students had pressures exceeding 130 mm. which he considered to be abnormal. According to Alvarez, the normal upper limit of the systolic blood pressure for healthy young men is 130 mm.

The lower limit of the normal systolic blood pressure, according to Norris, Bazett and McMillan (*loc. cit.*), is usually 110 mm., and when this pressure goes below 105 few can be on their feet regularly. It is evident that these figures cannot be applied to Indians.

*Diastolic pressure.*—The average diastolic pressure of  $71.8 \pm 5$  mm. in my subjects (Table III) is in keeping with the estimate of Woley (1910) who felt that it should be 70 per cent of the systolic pressure. Fisher, in the cases above referred to, found that the diastolic pressure had averaged 80 mm.

*Pulse pressure.*—Fisher, basing his results on the insurance statistics, above referred to, found that the average pulse pressure for all ages was 43.2 mm. This figure tallies closely with that of Symond (1923) who found the average pulse pressure for all builds and all ages to be 44.1 mm. Norris, Bazett and McMillan (*loc. cit.*) place the limits of normal pulse pressure between 30 mm. and 50 mm. and regard a pulse pressure persistently as low as 20 mm. or as high as 60 mm. as pathological.

The average pulse pressure in my subjects is  $28.6 \pm 7.2$  mm. which is lower than what Norris *et al.* (*loc. cit.*) regard as minimum in the normal range. The minimum pulse pressure obtained is 19 mm. in the case of subjects Nos. 24 and 28, and the maximum is 35.7 mm. in the subjects Nos. 3 and 6. These pulse pressures, though very low compared to the Western standards, are, however, quite in proportion to the systolic and the diastolic pressures obtained in the subjects.

TABLE III.

Subject number.	Date.	Mouth temperature, °F.	BLOOD PRESSURE.				PULSE RATE.			RESPIRATION RATE.	
			Systolic.	Average systolic.	Diastolic.	Average diastolic.	Average pulse pressure.	Before start of experiment.	During experiment.	Before experiment.	During experiment.
1	8-9-33	98.2	92	94.5	64	63	26.5	48	54	21	18
1	14-10-33	98.1	97		72			55	53	15	14
2	8-9-33	98.5	100	95.5	68	65.5	30.0	56	49	15	28
2	8-4-34	98.5	91		63			67	73	22	21
3	8-9-33	99.0	122		80			86	82	19	12
3	18-10-33	98.8	112	113	80	77.3	35.7	78	77	15	15
3	22-8-34	98.4	105		70			67	60	18	15
4	27-9-33	98.2	125	125.0	85	85.0	40.0	66	70	..	..
5	27-9-33	98.0	110	110	65	65	45	73	68	..	..
6	27-9-33	98.4	123		75			66	66	16	12
6	28-9-33	98.0	108	114.2	75	78.5	35.7	63	61	16	12
6	8-10-33	98.0	110		92			61	61	11	12
6	18-10-33	97.9	116		82			61	61	13	10
7	28-9-33	98.0	108	108	75	75	33	63	61	16	14
8	28-9-33	97.6	115	115	75	75	40	62	62	19	14
9	27-9-34	97.7	95	95	70	70	25	53	53	22	31
11	8-10-33	98.4	120		100			..	..	13	16
11	19-10-33	97.5	108	111	85	97	24	72	75	14	12
11	8-4-34	98.2	105		76			78	77	17	15
12	9-10-33	98.5	103								

13	12-10-33	97-5	102	102	102	72	72	72	30	65	60	20	17
14	12-10-33	99-4	98	98	98	68	68	68	30	93	93	22	21
15	14-10-33	98-7	110	110	110	72	72	72	38	78	82	19	17
16	15-10-33	98-5	111	111	111	70	70	70	35-5	54	54	15	15
16	31-7-34	98-0	95	95	95	65	65	65	35-5	54	53	18	15
16	1-8-34	98-0	..	..	..	..	..	..	35-5	53	51	20	16
17	21-10-33	98-0	95	95	95	65	65	65	30	58	58	20	14
18	18-10-33	98-4	85	85	85	64	64	64	21	60	62	20	21
19	24-3-34	98-4	97	97	97	73	73	73	24	61	60	17	11
20	25-3-34	98-0	98	98	98	67	67	67	31	57	57	17	14
21	31-7-34	98-4	100	100	100	75	75	75	25	60	61	18	15
21	1-8-34	98-2	..	..	..	..	..	..	25	61	60	18	17
22	27-9-34	98-2	75	75	75	60	60	60	15	51	53	21	21
23	28-8-34	98-2	105	105	105	75	75	75	30	67	65	15	12
24	28-8-34	98-4	97	97	97	78	78	78	19	65	60	20	19
26	22-8-34	98-0	85	85	85	65	65	65	20	52	51	19	14
26	23-8-34	97-6	..	..	..	..	..	..	20	47	44	17	16
27	9-10-34	99-0	84	84	84	64	64	64	20	79	71	20	17
28	9-10-34	99-0	89	89	89	70	70	70	19	75	74	20	18
29	12-10-34	98-4	98	98	98	73	73	73	25	71	67	21	20
30	12-10-34	98-2	112	112	112	78	78	78	34	85	81	22	13
31	27-10-34	97-6	95	95	95	75	75	75	20	56	57	15	9
32	27-10-34	98-4	105	105	105	75	75	75	30	65	68	23	18
AVERAGES ..		98-3	..	100-4	100-4	..	71-8	71-8	28-6	65	64	18-7	16-4
				± 11	± 11		± 5	± 5	± 7-2				



Previous observers in the tropical and sub-tropical countries have also noted low blood pressure in the inhabitants. McCay (1907) investigated the blood pressure in a large number of the Bengali students. The readings for the systolic blood pressure were taken at the disappearance of the pulse at the wrist. The pressure varied between 83 mm. and 118 mm., the average being slightly over 100 mm. (sitting position, arm level with heart). The observations on the Philippines by Musgrave and Sisson (1910) also indicate a lower systolic level. Cadbury (1922) studied the Chinese students and found their systolic blood pressure from 20 mm. to 30 mm. and diastolic pressure from 10 mm. to 20 mm. lower than the normal one finds in the inhabitants of North America and Europe.

Recent investigations in India give further evidence of low blood pressure in the Indians. Mukherjee and Gupta (*loc. cit.*) recorded the blood pressure of 12 Bengali students and found the average systolic to be 108 mm., average diastolic 78 mm. and the pulse pressure 30 mm. The *Indian Medical Record* (1931) gives the average systolic pressure of healthy Punjabees, between the ages of 21 and 25, as 114 mm. and the diastolic pressure as 74 mm. Stock and Karns (1924) examined 14 Indian students resident in London along with 103 British male students. They found a systolic average of 118.7 and a diastolic of 80.3, while the corresponding figures for the British group were 130.3 and 84.8 respectively. More recently, Subba Reddy (1933) has investigated the blood pressure in 112 male students of Vizagapatam Medical College between the ages of 18 and 27 years. His results show that 41.07 per cent of his subjects have the systolic pressure between 111 mm. and 120 mm., and 23.21 per cent between 121 mm. and 130 mm. The average systolic pressure was 116.8 mm., the average diastolic 76.9 mm., and the average pulse pressure 39.9 mm. These results are higher than those obtained by Cadbury in the Chinese students and higher than the results obtained in the case of my subjects. The high results obtained in Vizagapatam students may partly be due to the fact that the investigations were made in the afternoon presumably after the midday meal when the blood pressure may be expected to be somewhat higher. Even then the results obtained at Vizagapatam are lower than those given for Westerners by Fisher and Alvarez.

It is possible that the warmer climate of the tropical and sub-tropical countries lowers the blood pressure by causing the dilatation of the skin capillaries. That environmental conditions are an important factor in determining blood pressure is brought out by the studies of Tung (1928) on the Chinese. Blood pressure was examined in the case of 30 Chinese during their stay in America and also after their return to Peking. The average systolic pressure in America was found to be 113 mm. and in China 102 mm. The average diastolic pressure in America was 72, and it was 64 in Peking. Tung also reports that there is a distinct tendency for the blood pressures of Westerners resident in China to approach that of the Chinese. It therefore seems, as Tung suggests, that climatic differences are the main determining factors of blood pressure.

*Mouth temperature.*—This ranged between 97.5°F. and 99.0°F., the average working out as 98.3°F. (Table III). The mouth temperature was taken before the basal metabolism test was started, the subject lying on the metabolism cot. The thermometer was kept in the mouth for at least two minutes.

*Pulse rate.*—Column 9 of Table III gives the pulse rate taken just before starting the basal metabolism tests. This is compared with the rate of the pulse taken while the tests were in progress. It is seen that the former is slightly higher than the latter.

The pulse rate, taken before starting the metabolism tests, ranged between 53 in the case of subjects Nos. 9 and 16, and 86 in the case of subject No. 3. The average for 30 subjects works out as 65.

The data on the basal pulse rate are numerous in the case of Western subjects. Körösy (1910) counted the pulse rate of 255 soldiers, between the ages of 20 and 24 years, early in the morning before they had arisen from their beds and partaken of any food. He found the average rate of 64·2 with the maximum of 108 and the minimum of 42. Sutliff and Holt (1925) established for 146 adult men an average pulse rate of 62. Harris and Benedict (1919) (quoted by Boas and Goldschmidt, 1932) found the average basal pulse rate in the case of 121 adult males as  $61\cdot26 \pm 6\cdot73$ . Boas and Goldschmidt (*loc. cit.*) found in 51 males of the average age of 28·1 years, the basal pulse rate of  $61\cdot4 \pm 8\cdot22$ . They state that the basal pulse rate is lower and less variable than the heart rate measured under less standardized conditions, because many extra-cardiac factors that affect the heart rate are eliminated in the post-absorptive resting state.

It is seen that the pulse rate in the case of my subjects corresponds closely with the results obtained in the Westerners.

Mason (Mason and Benedict, 1931) also found that the average pulse rate in the case of the South Indian women corresponded closely with the average pulse rate in 90 American women studied by Harris and Benedict (*loc. cit.*).

That the pulse rate may differ with certain races is suggested by the observations of Steggerda and Benedict (*loc. cit.*) on the Mayas. They found in 30 male Mayas an average pulse rate of 52, with the minimum rate of 34—figures considerably lower than the average established in other peoples.

*Respiration rate.*—The respiration rate was counted before commencing the metabolism tests, the subject lying on the metabolism cot. Precautions were taken not to attract the subject's attention to his respirations. The respirations were counted for fully one minute. The lowest rate was in the subject No. 6, being 11, 13, 16 and 16 on the four different dates. The highest rate was 23 in the subject No. 32. The average for all the subjects works out as 18·7 per minute. There seems to be little difference between the respiration rates as obtained in these subjects and those described for Westerners. M'Kendrick (1889) mentions that in health there are usually 15 respirations per minute in the adult. Quetelet (quoted by M'Kendrick, *loc. cit.*) gives a table indicating the maximum respiration rate between the ages of 20 and 25 years as 24 per minute, the minimum 14 per minute and the average 18 per minute. According to Burton-Opitz (1921), the average rate of respiration between these ages is 18·7, exactly the same as has been obtained in the case of my subjects. Halliburton and McDowall (1930) give the rate of respiration in a healthy adult person as ranging from 14 to 18 per minute. Thus it would seem that, considering the warmer climate and the lower barometric pressure (about 720 mm. Hg.), the respiration rate in these subjects is not any higher than what has been recorded for Westerners.

Table III gives also the respiration rates during the periods in which the metabolism tests were carried out, i.e., the periods in which the subjects were connected with the metabolism apparatus. It is seen that in such a state the respiration rate is almost invariably lower, the average working out as 16.4. This accords with the experience of the Nutrition Laboratory of the Carnegie Institution of Washington that, not invariably but in general, there is a tendency for the respiration rate to become somewhat slower when any breathing appliance is attached (Shattuck and Benedict, *loc. cit.*; Mason and Benedict, 1931).

The respiration rates so obtained are not far from those found in the case of the Mayas by Shattuck and Benedict (*loc. cit.*) under similar conditions and which were not considered by them as far from the normal.

*Surface area.*—The necessity to obtain the surface area of the body has arisen in order to find the basal metabolism. A commonly accepted standard of the basal metabolism is the amount of heat produced per square metre of the body surface per hour. The surface area, however, was not actually measured but was obtained by the help of the excellent chart prepared by DuBois and DuBois (1916) (quoted by DuBois, 1927) and based on his height-weight formula.

In the case of the Chinese adults, it has been shown by Waddell, Han and Ch'en (1928) that the DuBois height-weight formula for the estimation of surface area is applicable with as much accuracy as in the case of the American subjects. Whether it can be applied with as much accuracy in the case of the Indians with smaller stem height is problematic.

*Oxygen consumption.*—This was measured with all possible care. All possible attention was given to guard against leakage. The mouthpiece gave no trouble, but the nose-clip required particular and, in some cases, constant attention. It had to be applied tight enough to prevent any leakage through the nose and at the same time not so tight as to be uncomfortable to the subject. The leak-tester was frequently used both before starting the chart to run to see if the nose-clip was properly applied or not, and also during the time when the chart was running and the respiration recorded. Occasionally it was found that the nose-clip seemed to have had been applied properly before starting a test, the leak-tester indicating no leakage; however, when the actual test was going on slight leakage was discovered during some period of the test. Such tests were obviously incorrect and were discarded. The leak-tester consisted of a brightly polished white metal piece. This was cooled by keeping it in cold water and, before use, was wiped clean with a piece of white muslin. During the monsoon season it was found that the use of ice-cold water to cool the leak-tester was not proper. This made the leak-tester too cold, so that when it was removed from the ice-cold water and wiped clean for use, it was found that a thin film of moisture from the atmosphere, which was nearly saturated, was deposited on the surface and made the leak-tester unsuitable for work. Water a few degrees colder than the tap-water was best for the purpose.

Usually two tests were made on a subject on any particular day. The oxygen consumption per minute was read off from the chart. This is shown in detail in column 6 of Table IV. It is seen that the results obtained of the two consecutive tests are usually within 5 per cent of each other. Column 7 of the same table gives the average of two or more tests done on the same day. Occasionally a test had to be discarded when a leak was discovered or when the breathing was very

TABLE IV.

Subject number.	Date.	Weight (without clothes), kg.	Height, cm.	Surface area, sq. m.	Oxygen consumption per minute, c.c.	Average oxygen consump- tion per minute, c.c.	HEAT PRODUCTION PER HOUR CAL.		DEVIATION FROM NORMAL.		
							Total.	Per sq. m.	Harris- Benedict.	Abb- DuBois.	Sanborn.
1	8-9-33	51.7	174.7	1.63	194	194	56.2	34.5	- 9	- 13	- 11
1	14-10-33	52.0	174.7	1.63	180, 177	178.5	51.65	31.3	- 20	- 21.2	- 18.5
2	8-9-33	58.5	171.2	1.69	207	207	59.9	35.5	- 9	- 10	- 10
2	8-4-34	55.4	171.2	1.65	206.5, 217.5	212	61.4	37.2	- 4.5	- 5.8	- 5.5
3	8-9-33	51.0	170.7	1.59	226	226	65.4	41.2	+ 7	+ 4.3	+ 5.0
3	18-10-33	51.0	170.7	1.59	205.0, 210.5	207.7	60.1	37.8	- 1	- 4.3	- 3.5
3	22-8-34	49.5	173.0	1.58	215.0, 216.0	215.5	62.35	39.5	+ 2	± 0	+ 0.2
4	27-9-33	56.2	170.7	1.66	211	211.0	61.1	36.8	- 5	- 6.8	- 5
5	27-9-33	47.2	167.9	1.52	205	205.0	59.3	39.5	- 1	- 1.2	- 1
6	27-9-33	56.0	172.5	1.67	245	245.0	70.9	42.5	+ 10	+ 7.6	+ 10

TABLE IV—*concl'd.*

No.	Date.	Weight (with clothes), kg.	Height, cm.	Surface area, sq. m.	Oxygen consumption per minute, c.c.	Average oxygen consump- tion per minute, c.c.	HEAT PRODUCTION PER HOUR CAL.		DEVIATION FROM NORMAL.		
							Total.	Per sq. m.	Harris- Benedict.	Aub. DuBois.	Sanborn.
6	8-10-33	55.8	171.5	1.66	212	212.0	61.4	36.98	- 4	- 6.4	- 4
6	18-10-33	55.8	171.5	1.66	233.0, 220.0	226.5	65.52	39.5	+ 2	± 0	+ 3.5
7	28-9-33	45.4	166.8	1.48	163.5	163.5	47.35	31.99	- 17.4	- 19	- 18
8	28-9-33	55.8	174.0	1.67	203.0	203.0	58.8	35.2	- 19	- 11	- 12
9	27-9-34	55.5	162.0	1.59	197.5, 193.0	195.2	56.5	35.5	- 9	- 10	- 8.7
10	17-8-33	59.0	166.4	1.67	190.0	190.0	55.0	32.93	- 14	- 16	- 14
11	19-10-33	55.0	175.0	1.67	205.0, 207.0, 214.0	208.7	60.4	36.2	- 8	- 11.7	- 9.3
11	8-10-33	54.0	175.5	1.66	224.5, 231.5	228.0	66.0	39.8	+ 2	- 3.0	- 1.5
11	8-4-34	56.0	175.5	1.69	236.5, 230.0	233.2	67.5	41.7	+ 3.5	+ 1.7	- 1.1
12	9-10-33	48.5	168.6	1.54	209.6	209.6	60.68	39.4	+ 1.5	± 0	± 0
12	21-10-33	48.5	168.6	1.54	173.5, 181.5	177.5	51.35	33.3	- 13.5	- 15.7	- 14.5
13	12-10-33	46.5	168.3	1.51	169.5	169.5	49.05	32.5	- 16.0	- 17.7	- 17.0
14	12-10-33	43.2	166.0	1.45	210.0	210.0	60.8	41.9	+ 8.0	+ 2.3	+ 8.0
15	14-10-33	45.5	168.6	1.49	199.0	199.0	57.6	37.9	- 1.0	- 4.5	- 3.0
16	15-10-33	52.0	166.6	1.57	209.5, 211.0	210.2	60.8	38.7	- 1.0	- 2.0	- 1.7
16	31-7-34	53.3	167.1	1.59	234.0, 231.0	232.5	67.35	42.35	+ 8.0	+ 7.2	+ 7.0

16	1-8-34	53.3	167.1	1.59	221.0, 219.0	220.0	63.7	40.0	+	2.0	+	1.5	+	1.5
17	21-10-33	49.0	160.2	1.49	181.0, 175.0	178.0	51.5	34.6	-	11.0	-	12.5	-	11.0
18	18-10-33	56.5	178.6	1.70	207.5, 215.5	211.5	61.25	36.0	-	8.0	-	8.8	-	11.0
19	24-3-34	52.5	170.0	1.60	207.0, 211.5	209.2	60.5	37.8	-	1.0	-	4.3	-	3.5
19	25-3-34	52.5	170.0	1.60	216.0, 203.0	209.5	60.65	37.9	-	1.0	-	4.0	-	3.5
20	24-3-34	71.3	177.5	1.88	232.0, 230.0	231.0	66.9	35.6	-	10.0	-	10.0	-	9.5
20	25-3-34	71.3	177.5	1.88	228.0, 228.0	228.0	66.0	35.1	-	11.1	-	11.0	-	11.0
21	31-7-34	53.0	177.0	1.65	199.0, 199.0	199.0	57.6	34.0	-	9.0	-	11.6	-	11.0
21	1-8-34	53.0	177.0	1.65	196.0, 186.0	191.0	55.3	33.5	-	14.0	-	17.7	-	14.5
22	27-9-34	55.5	174.50	1.67	207.5, 203.0	205.2	59.3	35.5	-	9.0	-	10.0	-	10.0
23	28-8-34	63.0	174.5	1.76	224.5, 217.5	221.0	64.0	36.4	-	8.0	-	7.6	-	8.3
24	24-8-34	57.0	161.0	1.62	184.0, 182.0	183.0	53.0	32.7	-	17.0	-	17.2	-	16.5
25	14-8-33	54.0	175.5	1.66	187.0, 191.0	189.0	51.7	33.0	-	10.0	-	16.4	-	10.2
26	22-8-34	57.2	162.0	1.61	214.0, 211.0	212.5	61.55	38.2	-	2.5	-	3.3	-	2.0
26	23-8-34	57.2	162.2	1.61	208.0, 202.0	205.0	59.3	36.8	-	6.0	-	7.0	-	5.5
27	9-10-34	46.5	164.5	1.50	209.0, 194.0	201.5	58.35	38.9	+	0.6	-	1.5	+	0.5
28	9-10-34	41.5	168.0	1.43	178.5, 168.0	173.2	50.1	35.0	-	11.0	-	14.6	-	7.0
29	12-10-34	56.2	172.0	1.66	197.5, 195.0	196.2	56.7	34.2	-	12.0	-	13.4	-	11.5
30	12-10-34	60.2	169.5	1.63	196.5	196.5	56.8	34.8	-	9.2	-	11.9	-	10.0
31	27-10-34	58.2	162.5	1.73	217.0, 218.5	217.7	63.0	36.4	-	6.5	-	7.8	-	6.5
32	27-10-34	68.2	167.0	1.76	228.5	228.5	66.15	37.5	-	6.0	-	4.9	-	4.0

irregular. Often tests on a subject were made on two or more different days. This was deemed especially necessary when high readings were obtained, to make sure that the high results were not due to any error in technique.

The average oxygen consumption for all the 32 subjects works out as 200·4 c.c. per minute.

*Heat production per hour.*—Column 8 of Table IV gives the total heat production of the body per hour from which the heat production per square metre of the body surface was calculated. This is given in column 9. The heat production was computed from the oxygen consumption. It was assumed that the average respiratory quotient was 0·82 in which case it has been calculated that each litre of oxygen gives rise to the production of 4·825 calories. For the purpose of expressing the amount of oxygen in terms of calories per hour, use was made of the excellent table contained in the Sanborn Company's handbook (Sanborn, 1933). The average heat production per square metre of the body surface per hour for all the 32 subjects works out as 36·16 calories.

*Basal metabolism.*—The basal metabolism is expressed here as the percentage deviation from two commonly accepted standards, viz., Aub-DuBois and Harris-Benedict. Comparison with the so-called Sanborn normals has also been made.

In order to compare the results obtained in the case of my subjects with the Harris-Benedict standards, I have made use of the tables published by the Carnegie Institution of Washington and abridged and re-arranged by Sanborn (*loc. cit.*).

The results are shown in Table IV. Here each day's result is given separately. In Table II is given the average result for each subject of the different days that the tests were taken. From this it is seen that only five of the 32 subjects give positive results. All the rest give negative results. The lowest results obtained are in the subjects Nos. 1, 13 and 24. The average for all the 32 subjects works out as 6·8 per cent lower than the Harris-Benedict standards and 8·7 per cent lower than the Aub-DuBois standards. The Aub-DuBois standards are, therefore, only 1·9 per cent higher than the Harris-Benedict standards so far as their application to these subjects is concerned.

According to Krogh's tables (1920), a uniform reduction of 6 per cent is made from Aub-DuBois standards for the ages of 15 to 75 years. It has been brought out by recent investigations that, for ages of 20 years and thereabout, the Aub-DuBois values are nearly the same as those of Harris-Benedict. The results obtained in my subjects tend to support this statement.

In the case of subjects Nos. 19, 20, 21 and 26, tests were made on two consecutive days. It is seen that, except in the case of subject No. 19, the second day's results are lower than the first day's results. This I do not believe to be due to a greater mental or muscular repose obtained on the second day, as these subjects were apparently quite at ease on the first day also. The lower results obtained on the second day are probably due to a feeling of fatigue as the result of experiments on two successive days. In the subjects Nos. 1, 2, 3, 6, 11, 12 and 16, tests were made on two or more widely different days. It is seen that the results of the second or subsequent tests are not necessarily lower than the first tests and also that the difference in the results is, sometimes, considerable. This might be due to the psychical state of the subject as has been recently brought out by Benedict (1935).

His own metabolism was measured on eighteen consecutive days in May 1932 and on thirty-three consecutive days in May and June 1933. The results were extraordinarily uniform from day to day. Only on five out of these fifty-one days there were marked variations which were attributed by the author to have been caused by emotional disturbances.

#### COMPARISON WITH THE BASAL METABOLISM RESULTS OBTAINED IN INDIA BY OTHER INVESTIGATORS.

It has already been stated that Mukherjee (*loc. cit.*) found, in 15 Bengalis, the basal metabolism 9 per cent lower than the Sanborn normals. And again, Mukherjee and Gupta (*loc. cit.*), studying 18 normal Bengali young men, obtained results 13·3 per cent below Aub-DuBois standards. Sokhey (*loc. cit.*) in Bombay obtained results 10 to 23 per cent below the Aub-DuBois standards, but as the details are not given, it is not possible to compare the present results with those obtained by Sokhey. Mason's and Benedict's (1931) results are the lowest of all, their 54 women subjects giving an average value of 16·9 per cent below Harris-Benedict standards and 17·2 per cent below those of Aub-DuBois. Banerji (1931) studied 145 prisoners of the district jail, Lucknow, and found the average basal metabolism to be 6·9 per cent below the 'European standards'. Banerji finds that during the monsoon season, when the atmosphere is very humid, the basal metabolism values are lower than those obtained during the drier seasons. He, however, does not mention which standards he had actually compared his results with. In any case it would be doubtful if the results obtained from prisoners in an Indian prison can be compared with the results obtained usually from students.

It is thus seen that the results obtained in my subjects are higher than those obtained in Bombay, Madras and Calcutta. They are about 5 per cent higher than the results obtained by Mukherjee and Gupta at Calcutta and about 9 per cent higher than the results obtained by Mason in the South Indian women. It may here be pointed out, however, that, so far, enough work has not been done in India to establish the sex difference in metabolism. It is quite possible, as DuBois (*loc. cit.*) suggests, that the sex difference is greater in the Oriental. This view is supported by the studies of Takahira who found the basal metabolism of the Japanese women 9·3 per cent less than that of the Japanese men : whereas the sex difference for the Americans has been worked out as about 7 per cent (Gephart and DuBois, 1916 ; Harris and Benedict, *loc. cit.*). Krishnan and Vareed (1932) studied 76 medical students in Madras of whom 15 were women. Their ages varied between 18 and 25 years. They found that the average basal metabolism of their male subjects was 12 per cent and of their female subjects 16 per cent below the DuBois standards. This gives a sex difference of 11 per cent.

It is thus seen that the results obtained by me at Hyderabad are higher than those obtained elsewhere in India with the exception of Lucknow. Nevertheless, these results are lower than the standards accepted for Westerners. The cause of this low metabolism in the Indians has been attributed to various factors, viz., climatic, dietetic, occupational and racial.

*Climatic factor.*—Is climate a factor in determining the basal metabolism ? This has already been discussed to some extent, but the evidence on this point is



conflicting. There is evidence, however, to believe that a prolonged residence in a warm climate tends to lower the basal metabolism. Earle (1928) reported the results of the studies made by him and his colleagues on 166 Chinese and also on 41 Westerners in Hong Kong and Peking. The basal metabolism of the Chinese subjects was found to be 8 per cent below the DuBois standards and that of Westerners in Hong Kong and Peking 7 per cent below the DuBois standards. On the other hand 5 Orientals examined by Earle in London were found to have basal metabolism 6.5 to 8.5 per cent below DuBois, Harris-Benedict and Dreyer standards. These results therefore 'appear to afford evidence of a climatic factor as the controlling influence in basal metabolism'.

In a recent publication Mason (*loc. cit.*) reports that the average decrease in metabolism in a group of nine European women moving to the tropics was 5.1 per cent and that three Indian women measured in two climates showed an increase in metabolism of 4.8 per cent in cold climates.

The influence of warm moist climate in lowering metabolism is suggested by Banerji (*loc. cit.*). If this is accepted, then it would also explain why the basal metabolism results are higher at Hyderabad than at the damp seaside places like Bombay, Calcutta and Madras. Table V gives the basal metabolism results with reference to the humidity of the air. It is seen that the average basal metabolism for those days in which the difference between the wet- and the dry-bulb readings was 4°C. or more works out as 4.4 per cent below the Harris-Benedict standards; whereas the average for those days in which this difference is less than 4°C. is 7.1 per cent below the Harris-Benedict standards. These experiments, however, are not of much value being few in number and not undertaken especially to show the effect of humidity on the basal metabolism. They, nevertheless, point to the possibility of humidity being a factor in modifying the basal metabolism. Further work is needed to elucidate this point.

TABLE V.

Subject num- ber.	Date.	Dry-bulb temperature, °C.	Wet-bulb temperature, °C.	DEVIATION FROM HARRIS- BENEDICT.	
				Per day.	Average.
2	8-4-34	31.5	24.5	- 4.5	- 4.5
11	8-4-34	32.0	24.5	+ 3.5	+ 3.5
19	24-3-34	28.5	20.0	- 1.0	} - 1.0
19	25-3-34	28.5	22.0	- 1.0	
20	24-3-34	29.5	21.0	- 10.0	} - 10.5
20	25-3-34	28.5	22.0	- 11.0	
27	9-10-34	29.0	25.0	+ 0.6	+ 0.6
28	9-10-34	29.0	25.0	- 11.0	- 11.0
31	27-10-34	25.0	19.5	- 6.5	- 6.5
32	27-10-34	26.5	21.0	- 6.0	- 6.0
AVERAGE					- 4.4

TABLE V--concl'd.

Subject num- ber.	Date.	Dry-bulb temperature, °C.	Wet-bulb temperature, °C.	DEVIATION FROM HARRIS- BENEDICT.	
				Per day.	Average.
1	8-9-33	..	..	- 9.0	} - 14.5
1	14-10-33	28.0	25.5	- 20.0	
2	8-9-33	..	..	- 9.0	- 9.0
3	8-9-33	..	..	+ 7.0	} + 2.7
3	18-10-33	27.0	25.5	- 1.0	
3	22-8-34	25.0	22.5	+ 2.0	- 5.0
4	27-9-33	..	..	- 5.0	- 1.0
5	27-9-33	..	..	- 1.0	} + 2.7
6	27-9-33	..	..	+ 10.0	
6	8-10-33	29.5	26.5	- 4.0	- 17.4
6	18-10-33	27.0	25.5	+ 2.0	- 10.0
7	28-9-33	..	..	- 17.4	- 9.0
8	28-9-33	..	..	- 10.0	- 14.0
9	9-9-33	26.5	25.0	- 9.0	} - 3.0
10	17-8-33	..	..	- 14.0	
11	8-10-33	28.5	26.5	+ 2.0	} - 6.0
11	19-10-33	26.5	25.0	- 8.0	
12	9-10-33	29.0	26.0	+ 1.5	- 16.0
12	21-10-33	24.5	22.0	- 13.5	+ 8.0
13	12-10-33	27.5	24.5	- 16.0	- 1.0
14	12-10-33	28.0	24.5	+ 8.0	} + 3.0
15	14-10-33	28.0	25.5	- 1.0	
16	15-10-33	28.0	25.5	- 1.0	- 11.0
16	31-7-34	25.0	24.0	+ 8.0	- 8.0
16	1-8-34	25.0	24.0	+ 2.0	} - 3.5
17	21-10-33	25.0	22.5	- 11.0	
18	18-10-33	27.0	25.5	- 8.0	- 14.0
21	31-7-34	25.5	24.5	- 9.0	- 17.0
21	1-8-34	25.0	24.0	+ 2.0	- 10.0
23	28-8-34	27.0	24.0	- 14.0	} - 4.2
24	28-8-34	27.2	24.2	- 17.0	
25	14-8-34	..	..	- 10.0	- 12.0
26	22-8-34	26.5	24.0	- 2.5	- 9.2
26	23-8-34	25.5	24.0	- 6.0	
29	12-10-34	28.0	25.0	- 12.0	
30	12-10-34	28.0	25.0	- 9.2	
AVERAGE					- 7.1

*Dietetic factor.*—Evidence has accumulated that protein-rich diet is a potent factor in raising the basal metabolism. This was brought forward by Krogh and Lindhard (1920). Heinbecker (1928) found the metabolism of Eskimos, who are heavy protein consumers, to average 33 per cent above the normal standards. Benedict and Roth (1915), however, examined 11 male and 11 female vegetarians and found that the metabolism did not differ significantly from that of the non-vegetarians. By vegetarians was meant persons who did not eat meat of any kind more than two or three times a year. These results were confirmed by Harris and Benedict (*loc.-cit.*).

Out of my 32 subjects, 8 are vegetarians. Out of these 8, Nos. 20 and 25 abstain totally from meat but take eggs occasionally. The rest of the 8, from Nos. 27 to 32 inclusive, abstain totally both from meat as well as eggs. These subjects belong to castes which have been vegetarians for generations. The average basal metabolism for all these 8 subjects works out as 8.08 per cent below the Harris-Benedict standards and 10.06 per cent below the Aub-DuBois standards. Vegetarian habits as such, therefore, seem to have a somewhat depressing effect on basal metabolism.

*Occupational factor.*—It is possible that the low metabolism rates obtained in India may, in part, be due to a less active life of the Indian subjects. Most of the tests made in India are on college students and it cannot be said with certainty that the college students in India lead a less active life than students in colder climates. It is only surmised that the warmer climate of India would lead to a less active life. That athletic training raises the rate of the basal metabolism has been observed by many workers (Benedict and Smith, 1915; Gephart and DuBois, *loc. cit.*; Lauter, 1926; etc.) Against this, Tilt (1930) found the basal metabolism of 10 athletic women in Florida to be no higher than that of the whole mixed group. And Krishnan and Vareed (*loc. cit.*) found that in the case of 6 of their subjects, 'who were daily indulging in fairly severe exercise', the basal metabolism was distinctly lower than that of the mixed group.

*Racial factor.*—In 1925, MacLeod, Crofts and Benedict (*loc. cit.*) emphasized for the first time the existence of a racial factor. They examined 7 Chinese and 2 Japanese women students in America and found their basal metabolism to be 10.4 per cent below the Harris-Benedict standards. As these standards have been considered to be 5 per cent too high for American college women, this leaves the Oriental women students only 5 per cent below their American colleagues. This difference was considered to be due to the existence of a racial factor. Subsequently the Nutrition Laboratory of the Carnegie Institution of Washington has embarked on a comprehensive programme for the study of the basal metabolism of various races. From the results obtained, Benedict and his co-workers of the Nutrition Laboratory have come to a definite conclusion that a racial factor exists. There are, however, difficulties in the unqualified acceptance of this conclusion.

An important series of investigations has been carried out on the Mayas of Yucatan. On three different expeditions it was found that the basal metabolism in the Mayas was definitely higher than in the case of the North American whites. And, what is more, there was a definite suggestion that a Maya of purer extraction tended to have a higher metabolism than one less pure. This, however, has not been definitely settled. Recently, Pi-Suner (1933) has obtained similar results on another aboriginal tribe, the Mapuche Indians of southern Chile. In 31 of his male subjects he found an average basal metabolism 9.8 per cent higher than the Harris-Benedict standards. The Nutrition Laboratory workers have contracted the high basal metabolism of the Maya males with the very low basal metabolism of the South Indian females and conclude that such a wide difference cannot but be due to a racial factor.

There is, however, reason to believe that in the case of both the Mayas and the Mapuches there are other factors which would tend to raise the basal metabolism. Both the Mayas and the Mapuches are aboriginal tribes. The living conditions

amongst them are very poor. 'The Maya sleeps inadequately protected by his meagre clothing and his hammock against the cold nights'. The same may be said of the Mapuches of southern Chile where the temperature goes down in September (when the above-mentioned observations were made) to the freezing point. It is quite possible that in the case of these aboriginal tribes the higher basal metabolism is a result of a compensatory mechanism to offset the effect of exposure to cold nights.

Recently Benedict and Meyer (*loc. cit.*) have examined 18 American-born Chinese girls (12 to 22 years of age) living in the United States. They find that these girls have a basal metabolism averaging 9.2 per cent lower than that of the American girl scouts of the same ages and 6.1 per cent lower than the Aub-DuBois standards for American girls. Now if it is conceded, as has been suggested, that the Aub-DuBois standards are 5 per cent too high even for American women then these Chinese girls have metabolism averaging practically the same as Aub-DuBois predictions. On the other hand the metabolism of the girl scouts was taken while they were asleep. It is therefore doubtful whether it is quite fair to apply the girl scouts' standards (with 10 per cent correction for sleep) in the case of the Chinese. Besides this, the authors admit that these American-born Chinese girls were not free from Chinese dietetic habits. Under the circumstances it cannot be said that these tests definitely establish the existence of a racial factor. In the words of the authors themselves, 'until further measurements are made upon other girls (both Chinese and Caucasians) in this age range, however, the drawing of any final conclusion must be held in abeyance'.

If we now review the numerous observations that have been made on different races in different countries we can have some idea of how far the racial factor operates. The following are some of the main observations:—

Observations.	DEVIATION FROM NORMAL STANDARDS.	
	Harris-Benedict.	Aub-DuBois.
1. Eijkmann's observations on the Malays in Batavia.	..	— 0 per cent.
2. Takahira's observations on the Japanese men.	..	— 5.5 "
3. Earle's observations on the Chinese.	..	— 8.0 "
4. Mason's observations on the Madrascce women.	— 16.9	— 17.2 "
5. Mukherjee and Gupta's observations on the Bengali young men.	..	— 13.3 "
6. Observations on the Hyderabadi young men.	— 6.8	— 8.7 "

These figures may be compared with the following figures obtained on the Europeans in the East :—

Observations.	DEVIATION FROM NORMAL STANDARDS.	
	Harris-Benedict.	Aub-DuBois.
1. Montoro's observations on the white Cubans in Havana (quoted by DuBois, 1927).	— 15·8	— 15·5 per cent.
2. de Almeida's observations on white men in Brazil.	— 13·6	— 16·2 „
3. Mason's (1934) observations on the European women in Madras.	— 7·9	— 12·5 „
4. Earle's report on European men at Hong Kong and Peking.	..	— 7·0 „

The comparison of the two sets of figures shows how closely they resemble each other. Had the racial factor been an important factor in determining the basal metabolism, it would seem strange that the basal metabolism figures of races so widely different as the Caucasians of Europe and America and the Mongolians of China and Japan should tally so closely. It would be more reasonable to suppose that though a racial factor may exist it is probably not so important as the climatic and the environmental factors in determining basal metabolism.

#### CAUSE OF THE LOW BASAL METABOLISM IN THE INDIANS.

Very few observations have so far been made in India and these go to show the existence of a lower basal metabolism in the Indians. Probably all the causes discussed above operate in bringing about this low level. Mukherjee and Gupta (*loc. cit.*) have attributed it to climatic and nutritional causes. McCarrison (1927) studied the dietary habits of the different Indian provinces and found that the Bengali, the Kanaree and the Madrassie diet is of low biological value and is poor in the contents of suitable proteins, vitamins and mineral elements. The lowest figures have been obtained in the Madrassie women. But if a 5 per cent correction is made to these figures on the ground that the Aub-DuBois and the Nutrition Laboratory predictions are 5 per cent too high for women, then the average works out as 11 to 13 per cent which is about the same as for the Madrassie men.

Mason and Benedict (1931) attributed the low metabolism of the Madrassie women to the existence, at least in part, of a definite racial factor. Other possible factors which they suggest are : a low protein diet, tropical conditions of climate and a state of relaxation during repose as complete as that which is found during sleep among Westerners.

In a later publication Mason and Benedict (1934) give the results of their investigations of the basal metabolism in 7 Madrassese women while awake and during sleep, and have compared this result with that found in 2 Western women living in Madras under the same conditions. They found that in the Madrassese women the oxygen consumption during sleep decreased on an average 9·8 per cent. The two Western women gave similar results. The authors conclude that the state of relaxation is not the causal factor in the low basal metabolism of the Madrassese women. This conclusion seems to be justified, however, only if it can be proved that the amount of muscular relaxation during sleep is the same in both the Oriental and the Occidental.

#### SUMMARY AND CONCLUSIONS.

Basal metabolism tests and physical character studies have been carried out in 32 young men at Hyderabad (Deccan). The ages varied from 19 to 31 years, the average being 22 years.

No difference was found in the pulse and the respiration rates as compared with the Western standards.

The subjects were found to have somewhat (about 1 cm.) shorter stem height and correspondingly longer legs compared to the measurements obtained in the Westerners.

A pelidisi of about 92 indicates the normal state of nutrition in young Indians, the average pelidisi for 30 out of the 32 subjects being  $92 \pm 4$ .

The blood pressure was found to be considerably lower compared to the Western standards. The average systolic blood pressure in 30 out of the 32 subjects was  $100\cdot4 \pm 11$  mm., the average diastolic pressure  $71\cdot8 \pm 5$  mm., and the average pulse pressure  $28\cdot6 \pm 7\cdot2$ .

The basal metabolism was found to be 6·8 per cent below the Harris-Benedict and 8·7 per cent below the Aub-DuBois standards. The factors which may tend to bring about this low level of metabolism are discussed.

The possibility of the humid climate being a factor in lowering metabolism is suggested.

Eight of the subjects who were vegetarians showed the basal metabolism on an average about 2 per cent lower than that obtained for the whole mixed group.

For height and age, the weight in this group of subjects was less than the American standards.

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## FACTORS AFFECTING THE CAROTENE CONTENT OF CERTAIN VEGETABLE FOOD-STUFFS.

BY

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IN a previous communication (De, 1936), it was pointed out that non-leafy vegetable foods, such as roots, pulses and fruits, do not lose carotene (provitamin A) appreciably during a reasonable period of storage. In certain foods, indeed, development of carotene may continue until long after harvesting; ultimately, however, deterioration takes place. The green leafy vegetables, which are very rich in carotene, were found to lose a high proportion of their carotene even during a few days' storage under ordinary conditions. The recognition of the importance of vitamin A in the feeding of live stock and poultry has resulted in a considerable amount of work on the variation of the vitamin A values of alfalfa hays and meal (Hauge and Aitkenhead, 1931; Russell, 1929; Guilbert, 1935), but little similar work has been done on human foods. Guilbert (*loc. cit.*), in his extensive work on alfalfa hays and meal, using a colorimetric method of assaying carotene, investigated the influence of various factors, viz., light (visible and ultra-violet), temperature, bacterial and enzyme action, on the carotene content of samples during storage. Little or no loss of carotene occurred during vacuum-drying even at a temperature of 100°C. Guilbert's figures show that the rate of destruction of carotene in dried alfalfa during storage was negligible as compared to that occurring in fresh samples.

Both vitamin A and carotene are known to be stable to heat of ordinary degree in the absence of oxygen. Yellow corn, carrot, sweet potato and squash have been heated for 3 hours at 250°F. with no loss of vitamin A potency. The potency of most canned foods was found to be practically undiminished (Sure, 1933). According to Steenbock and Boutwell (1920), Coward and Morgan (1935) and many others, vegetable foods do not lose appreciable amounts of vitamin A in the domestic cooking or canning processes.

Germination of grains like corn, barley and mung has not been found to produce any noticeable variation in their vitamin A potency; an increased potency was, however, noted when the germinated seeds formed leaves (Karshan, Krasnow and Harrow, 1927; Coward and Drummond, 1921; Miller and Hair, 1928).

In most of the work quoted above, the vitamin A value of test materials was estimated by feeding experiments. It is now established that the vitamin A potency of vegetable food-stuffs is mainly due to carotene (precursor of vitamin A), and there have already been introduced several laboratory methods, chemical or physical, for assaying carotene which probably give more accurate results than any that can be obtained by biological tests.

In the present work the variation of carotene in a few edible leafy vegetables during storage under various conditions was studied, using the spectrophotometric method previously described (De, 1935, 1936). Simultaneously the influence of other factors, viz., milling, parboiling, cooking and sprouting, was also investigated.

#### EXPERIMENTAL.

The various factors, the influence of which on carotene content was studied, are described in Tables I to V. The leafy vegetables were obtained fresh from a neighbouring garden and immediately used for experiment; the other test materials were bought in the local market. As far as possible, identical specimens of the same material were used throughout each series of experiments. Results are expressed in  $\gamma$  (0.001 mg.) per gramme of initial weight.

TABLE I.

*Effect of various factors (parboiling, husking, light, temperature, bacterial action, etc.) on the carotene content of certain vegetable food-stuffs.*

Test No.	Treatment.	Carotene content per gramme of initial weight in $\gamma$ (0.001 mg.).	Percentage loss of carotene.
1	Unmilled raw rice, husk removed from paddy by hand	0.34	..
	Unmilled parboiled rice, husk removed from paddy by hand.	0.26	23.5
	Raw rice, from same paddy, home-pounded ..	0.04	90.0
	Parboiled rice, from same paddy, home-pounded ..	0.15	55.5
	Highly milled rice (different sample) .. ..	Nil	100.0
2	Amaranth ( <i>Amaranthus gangeticus</i> ), fresh leaves ..	68.16	..
	Leaves preserved in dark in a vacuumized desiccator for 2 days at 0°C.	65.10	4.5
	do. do. for 2 days at 15°C. to 22°C.	64.30	5.7
	Leaves preserved in paper packets for 2 days at 15°C. to 22°C. (room temperature).	61.60	9.6
	do. do. 3 days at 15°C. to 22°C.	54.12	20.6
	Leaves exposed to diffused sunlight in the laboratory for 2 days.	51.70	24.1
	do. do. for 3 days ..	44.00	35.4

TABLE I—*contd.*

Test No.	Treatment.	Carotene content per gramme of initial weight in $\gamma$ (0.001 mg.).	Percentage loss of carotene.
	Leaves kept under water containing a little formaldehyde for 3 days at 15°C. to 22°C. (remained green and fresh).	63.34	7.1
	Leaves kept under water for 3 days at 15°C. to 22°C. (remained green and fresh).	49.60	27.2
	Leaves kept under water containing toluene for 24 hours at 15°C. to 22°C. (greenness and freshness lost).	40.60	40.4
3	Amaranth, fresh leaves .. .. .	61.60	..
	Leaves preserved under water containing a little formaldehyde for 24 hours at 38°C. (remained green and fresh).	59.70	3.1
	do. with toluene for 24 hours at 38°C. (greenness and freshness lost).	17.60	71.3
	Leaves kept under water containing formaldehyde for 3 days at 38°C.	56.60	8.1
	Leaves kept under water alone for 3 days at 38°C.	28.00	53.6
	Leaves kept in paper packets for 3 days at 38°C. ..	35.55	42.5
4	Celery leaves, fresh .. .. .	74.70	..
	Leaves stored in a vacuumized desiccator at room temperature for 24 hours.	73.14	2.1
	do. do. for 2 days .. .. .	70.40	5.8
	Leaves preserved in paper packets in the laboratory at room temperature for 5 days.	45.70	32.8
	Leaves preserved under water containing formaldehyde for 5 days at room temperature.	66.40	11.1
5	Coriander ( <i>Coriandrum sativum</i> ), fresh leaves ..	116.70	..
	Leaves preserved under water containing a little toluene at room temperature for 24 hours.	64.80	44.6
	Leaves preserved in an atmosphere of CO <sub>2</sub> gas under water containing a little toluene at room temperature for 2 days.	105.00	10.0
	Leaves preserved under water containing a little formaldehyde at room temperature for 4 days.	99.00	15.2
	Leaves preserved in paper packets at room temperature for 4 days (became dried up and unfit for use).	70.00	40.0
	Leaves sprinkled with water and preserved at room temperature wrapped in green plantain leaf for 2 days (remained quite green and fresh).	112.00	4.0
	do. do. for 4 days (part became rotten; rest remained green and fresh).	70.00	40.0
	Leaves sprinkled with water and preserved at room temperature (wrapped in wet cloth) for 2 days.	110.00	5.7
	do. do. for 4 days (part became rotten; rest remained green and fresh).	72.00	38.3

TABLE II.

*Effect of storage at various temperatures on the carotene content of coriander leaves (Coriandrum sativum).*

Test No.	Treatment.	Carotene content per gramme of initial weight in $\gamma$ (0.001 mg.).	Percentage loss of carotene.
1	Leaves, fresh .. .. .	103.6	..
	Leaves, vacuum-dried at 100°C. .. ..	104.0	..
2	Leaves preserved in paper packets in the laboratory for 5 hours at 0°C.	102.0	1.5
	do. at 15°C. to 22°C. (room temperature) ..	100.2	3.3
	do. at 38°C. .. .. .	94.3	8.9
3	Leaves preserved in paper packets in the laboratory for 24 hours (1 day) at 0°C.	99.0	4.4
	do. at 15°C. to 22°C. .. .. .	88.7	14.4
	do. at 38°C. .. .. .	82.0	20.8
4	Leaves preserved in paper packets in the laboratory for 2 days at 0°C.	90.0	13.1
	do. at 15°C. to 22°C. .. .. .	75.0	27.6
	do. at 38°C. .. .. .	65.0	37.3
5	Leaves preserved in paper packets in the laboratory for 3 days at 0°C.	79.5	23.3
	do. at 15°C. to 22°C. .. .. .	68.6	33.8
	do. at 38°C. .. .. .	57.6	44.4
6	Leaves preserved in paper packets in the laboratory for 5 days at 0°C.	68.7	33.7
	do. at 15°C. to 22°C. .. .. .	57.7	44.3
	do. at 38°C. .. .. .	45.4	56.2
7	Leaves preserved in paper packets in the laboratory for 12 days at 0°C.	61.5	49.6
	do. at 15°C. to 22°C. .. .. .	49.8	51.9
	do. at 38°C. .. .. .	40.5	60.9

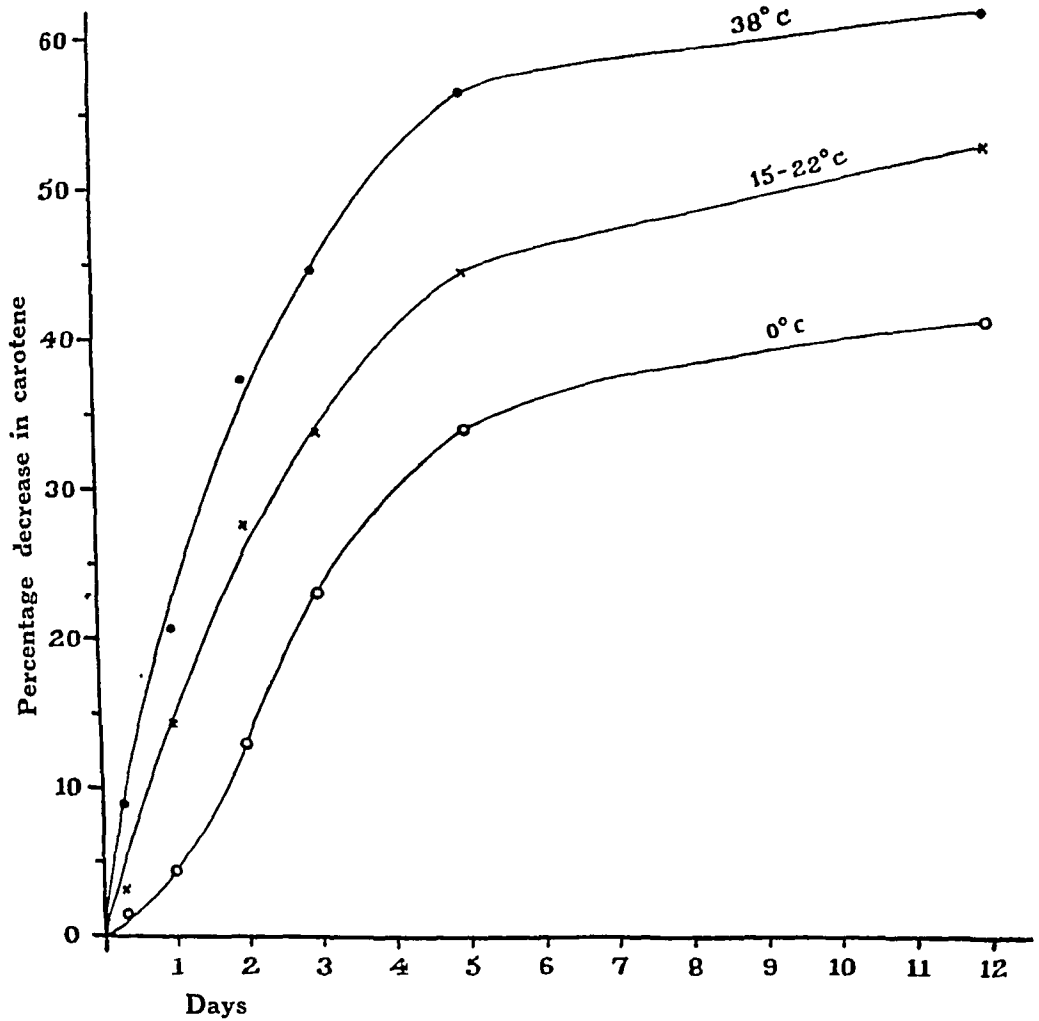


FIG. 1. Percentage decrease in the carotene content of coriander leaves stored in paper packets in the laboratory at 0°C., 15°C. to 22°C., and 38°C., respectively.

TABLE III.

*Effect of storage at various temperatures on the carotene content of spinach leaves.*

Test No.	Treatment.	Carotene content per gramme of initial weight in $\gamma$ (0.001 mg.).	Percentage loss of carotene.
1	Fresh leaves .. .. .	34.84	..
	Leaves, vacuum-dried at 100°C. .. ..	34.71	..
2	Leaves preserved in paper packets in the laboratory for 5 hours at 0°C.	33.50	3.8
	do. at 15°C. to 22°C. (room temperature) ..	32.70	6.1
	do. at 38°C. .. ..	29.88	14.2
3	Leaves preserved in paper packets in the laboratory for 24 hours at 0°C.	32.30	7.3
	do. at 15°C. to 22°C. .. ..	28.35	18.6
	do. at 38°C. .. ..	22.70	34.8
4	Leaves preserved in paper packets in the laboratory for 2 days at 0°C.	29.20	16.2
	do. at 15°C. to 22°C. .. ..	24.00	31.1
	do. at 38°C. .. ..	20.10	42.3
5	Leaves preserved in paper packets in the laboratory for 3 days at 0°C.	27.40	21.4
	do. at 15°C. to 22°C. .. ..	22.40	35.7
	do. at 38°C. .. ..	18.30	47.5
6	Leaves preserved in paper packets in the laboratory for 5 days at 0°C.	24.00	31.1
	do. at 15°C. to 22°C. .. ..	18.70	46.3
	do. at 38°C. .. ..	16.20	53.5

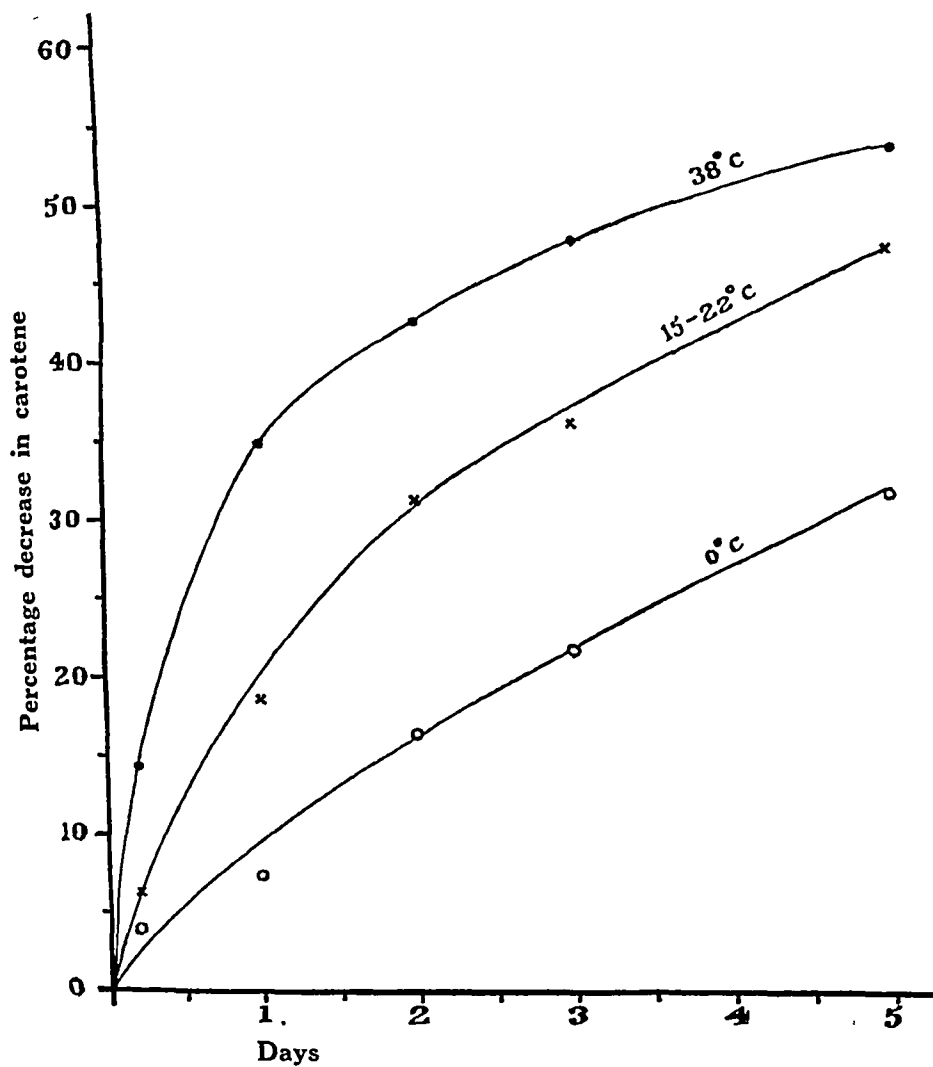


Fig. 2. Percentage decrease in the carotene content of spinach leaves stored in paper packets at 0°C., 15°C. to 22°C., and 38°C., respectively.



TABLE IV.

*Effect of cooking on the carotene content of certain vegetable foods.*

Test No.	Treatment.	Carotene content per gramme of initial weight in $\gamma$ (0.001 mg.).
1	Amaranth leaves ( <i>Amaranthus gangeticus</i> ) ..	71.70
2	do. do. boiled in tap-water for 30 minutes ..	72.90
3	do. do. for 1 hour ..	70.40
4	Spinach leaves .. ..	38.80
5	do. boiled for 1 hour .. ..	38.50
6	Potato .. ..	0.28
7	do. boiled for 1 hour .. ..	0.26
8	Lentils ( <i>Lens esculenta</i> ) .. ..	0.53
9	do. boiled for 1 hour .. ..	0.31
10	Red gram ( <i>Cajanus indicus</i> ) .. ..	2.11
11	do. boiled for 1 hour .. ..	1.43
12	Unpolished parboiled rice .. ..	0.26
13	do. do. boiled for 45 minutes ..	Nil or trace.
14	Ragi ( <i>Eleusine coracana</i> ), powdered ..	0.61
15	Ragi, powdered, boiled for 45 minutes ..	0.26

TABLE V.

*Effect of sprouting on the carotene content of certain pulses.*

Test No.	Treatment.	Carotene content per gramme of initial weight in $\gamma$ (0.001 mg.).
1	Bengal gram ( <i>Cicer arietinum</i> ) .. ..	5.90
2	do. sprouted for 2 days in tap-water (no shoots visible).	5.50
3	do. sprouted for 4 days in tap-water (shoots were visible).	4.80
	do. for 5 days .. ..	4.03
	do. " 6 " .. ..	3.75
	do. " 7 " .. ..	3.40
	do. " 10 " .. ..	2.80
	do. " 15 " .. ..	2.00
2	do. sprouted for 4 days in 50 mg. per cent $MnCl_2$ solution (shoots were visible).	3.70
	do. do. for 5 days .. ..	3.30
3	do. sprouted for 4 days in 50 mg. per cent $MgCl_2$ solution (shoots were visible).	2.10
4	Green gram ( <i>Phaseolus radiatus</i> ), whole grains ..	4.70
	do. sprouted in tap-water for 1 day ..	3.52
	do. do. 2 days ..	2.15
	do. do. 3 " ..	1.95
	do. do. 5 " ..	1.45
	do. do. 7 days (growth of leaf first visible).	3.52
	do. do. 9 days (leaf visible) ..	8.80

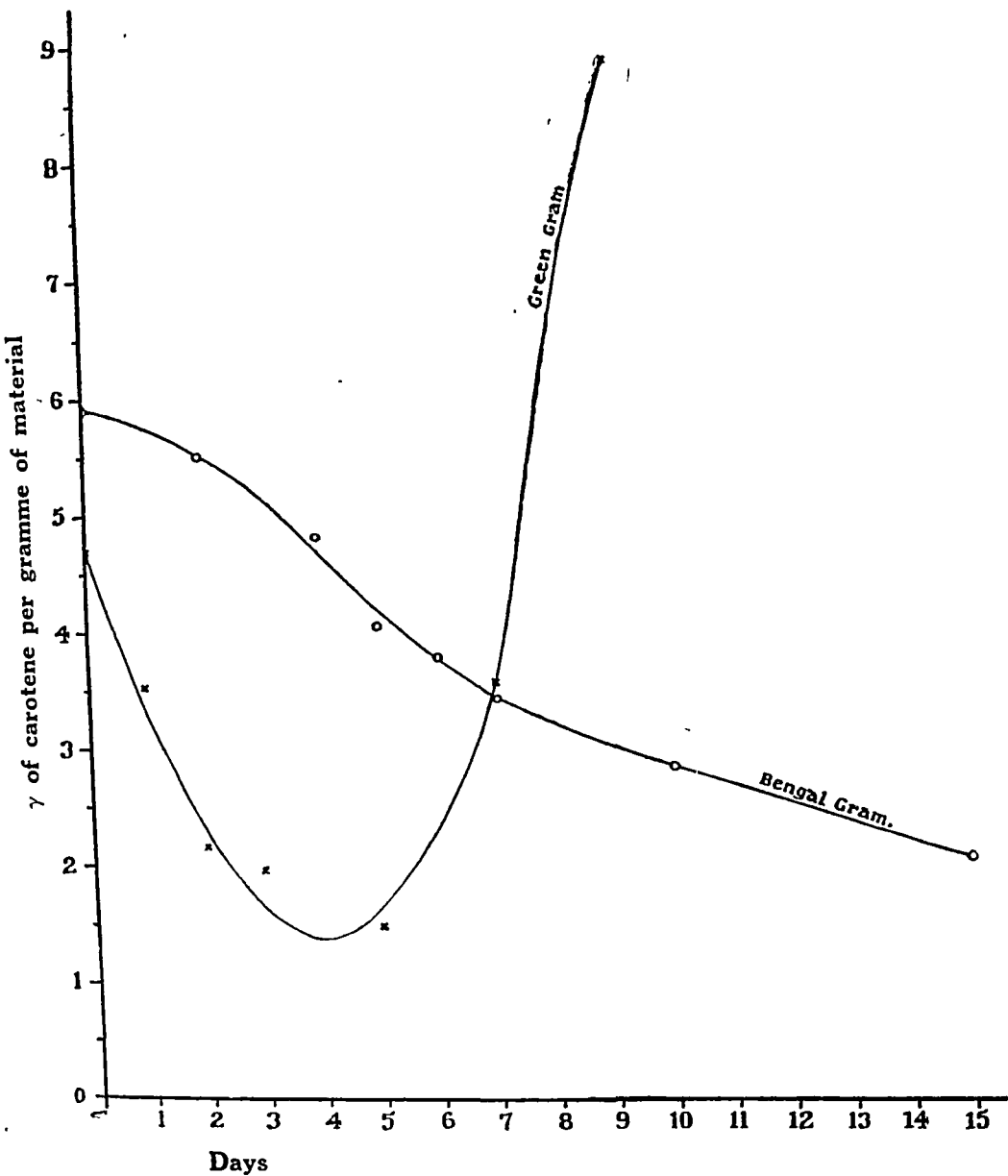


FIG. 3. Carotene content of Bengal gram and green gram sprouted in tap-water for a varying number of days.

## DISCUSSION AND SUMMARY.

The process of parboiling causes a slight loss of carotene, which becomes evident when similar samples of unmilled raw and parboiled rice, husked by hand, are compared. 'Home-pounding', which removes most of the germ and a certain proportion of the pericarp, results in a loss. It is noticeable that the carotene content of the home-pounded parboiled rice was superior to that of the home-pounded raw rice, in spite of the initial loss of carotene in the former during the process of soaking, steaming and sun-drying. This is due to the fact that it is easier to separate the husk from parboiled paddy in which the husk is split, than from raw paddy; in the case of the latter more prolonged and vigorous pounding is necessary, the grains are broken, and there is a greater loss of germ and pericarp. Highly milled rice was found to be completely devoid of carotene.

No loss occurred in vacuum-drying of coriander and spinach leaves at 100°C., a finding in agreement with the well-known fact that carotene is relatively stable to heat in the absence of air. Comparatively rapid loss of carotene occurred from exposing fresh amaranth leaves to diffused sunlight. Fresh amaranth leaves preserved under water with a little formaldehyde at 38°C. lost 3 per cent of carotene in 24 hours and 8 per cent in 3 days. Slightly less destruction was noted under similar conditions at room temperature. Leaves preserved under water with a little toluene at 38°C. lost 71 per cent in 24 hours; a 40 per cent loss was noted under similar conditions at room temperature. Guilbert (*loc. cit.*) noted a 95 per cent loss of carotene in incubated alfalfa under water with a little toluene, and ascribed this rapid destruction to strong enzyme action. The results obtained here, however, lead to a different view. The purpose of adding toluene or formaldehyde is to prevent mould or bacterial action. Toluene, being a fat solvent, liberates free carotene from the stored material, and the freed carotene becomes subject to atmospheric oxidation. It was found that destruction of carotene in coriander leaves preserved in water containing toluene was largely prevented in an atmosphere of CO<sub>2</sub> gas. In the case of samples preserved with formaldehyde, no carotene is liberated; consequently only a little destruction takes place. It appears that enzyme action can be only a minor factor in causing destruction of carotene in incubated samples. Amaranth leaves kept under water alone for 3 days lost 27 per cent and 54 per cent of carotene at room temperature (15°C. to 18°C.) and 38°C. respectively, while samples preserved under water with a little formaldehyde lost very little in the same time (7 per cent to 8 per cent). The carotene content of food-stuffs in storage is probably considerably affected by bacterial action.

3 studies on destruction of carotene in coriander and spinach leaves  
 4 are at various temperatures are presented in Tables II and III; and  
 Figs. 1 and 2. It will be seen that the leafy vegetables lose carotene  
 under ordinary conditions at all temperatures, the rate of loss being  
 greater at higher temperatures. The modern domestic frigidaire thus to some extent  
 maintains the vitamin A potency of vegetables, etc. From the shape of the curves  
 the rate of destruction gradually decreases as the materials become  
 colder. Alfalfa leaves can be stored for several weeks with little loss of  
 carotene (*loc. cit.*); fresh leaves preserved in paper packets lost  
 little carotene under similar conditions, while samples preserved *in vacuo*

were little affected. These observations lead to the view that mere dehydration in presence of air destroys carotene during storage.

Green vegetables, sprinkled with water and preserved by being wrapped in green plantain leaves or wet cloth, retain a high carotene content for several days. It is interesting to note that this is the usual method employed by the public in India for preserving the freshness of leafy vegetables.

The results of the studies on the effect of cooking are set out in Table IV. The materials were boiled from cold in tap-water for the stated periods. It will be seen that the leafy vegetables and potatoes lost little or no carotene on boiling, while the legumes lost appreciably. The sample of rice was found to be devoid of carotene as a result of cooking for 45 minutes. The destruction of carotene in cooked lentils, red gram, and rice can be accounted for by the fact that the materials on boiling went into solution in the water (in the case of rice the superficial layers of the grain dissolved in the water and the cooked grains became white); the broken tissues were thus easily attacked by heat and atmospheric oxidation.

Table V records data on the effect of sprouting. Several 5-gramme samples of Bengal gram and green gram were kept soaked in tap-water in Petri dishes for 24 hours; afterwards they were spread on separate moist filter-papers which were kept wet by sprinkling water on them occasionally. Green gram showed sprouts after 24 hours, while Bengal gram took over 3 days. The carotene content of the samples was investigated after various periods of sprouting. During the period of observation, Bengal gram sprouts did not form leaves and there was gradual loss of carotene. Similarly green gram showed gradual loss during certain periods of sprouting; when, however, the formation of leaf was visible carotene content showed a sudden rise. The results are set out graphically in Fig. 3.

Dutcher, quoted by Haber and Swanson (1935) in an unpublished investigation, noted that spinach grown in soil containing insufficient amounts of manganese manufactured less vitamin A (carotene) than did spinach grown in the presence of adequate amounts of that element. Magnesium also seems to play some rôle in the formation of plant pigments (Ewart, 1917). Tests Nos. 2 and 3, Table V, show that manganese and magnesium chloride have no marked influence on the carotene content of germinated seeds. The gradual loss of carotene in the pulses during the course of germination may possibly be due to oxidation (bacterial, enzymic or otherwise) or carotene may be used up in the growth of the sprouts of the germinated seeds. The latter inference involves the view that young plants, like young animals, need vitamin A, in the form of carotene, for their growth. Virtanen and his associates (1933), conducting studies on vitamin formation in plants, came to this conclusion. Direct evidence in support of this view is, however, lacking. Whether inorganic salts have any specific effect on the synthesis of carotene, and whether carotene is actually needed for the growth and development of young plants, remains a field for investigation.

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## MIGRATION OF ASCORBIC ACID (VITAMIN C) IN AN ELECTRICAL FIELD.

BY

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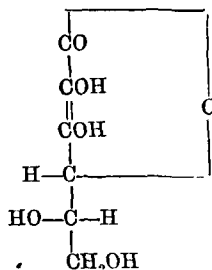
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THE consensus of opinion on the constitution of ascorbic acid, based on both analytical and synthetic studies, is that it has a lactone structure. Its structural formula is:—



It is apparent that no free carboxyl group is present, contrary to the views originally held by certain workers. It is known that lactones are usually broken up in strong alkaline media. In the present investigation the electrical migration properties of ascorbic acid were studied, with the object of discovering whether it behaves as a typical lactone and at what pH this lactone breaks up.

### EXPERIMENTAL.

The apparatus used in the present studies is shown in the accompanying Diagram. It consists of a central chamber A, of about 15 c.c. capacity, containing the solution of ascorbic acid in the respective buffers (usually 30 mg. of ascorbic

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acid in about 10 c.c. of the buffer). The central chamber is connected to B and C, the positive and negative electrode vessels, containing the buffer, through side arms provided with taps. The electrode vessels are connected in series to a sodium chloride vessel and a copper sulphate vessel (10 per cent NaCl and  $\text{CuSO}_4$  solutions respectively) by means of agar bridges. Two stout copper electrodes dip into the copper sulphate solution. A galvanometer is introduced into the circuit, as shown in the Diagram. The electrodes, being non-polarizable, permit a steady flow of current over long periods. The current is of the order of 3 to 6 milliamperes at 240 volts; in these experiments it was usually passed for about 24 hours. The

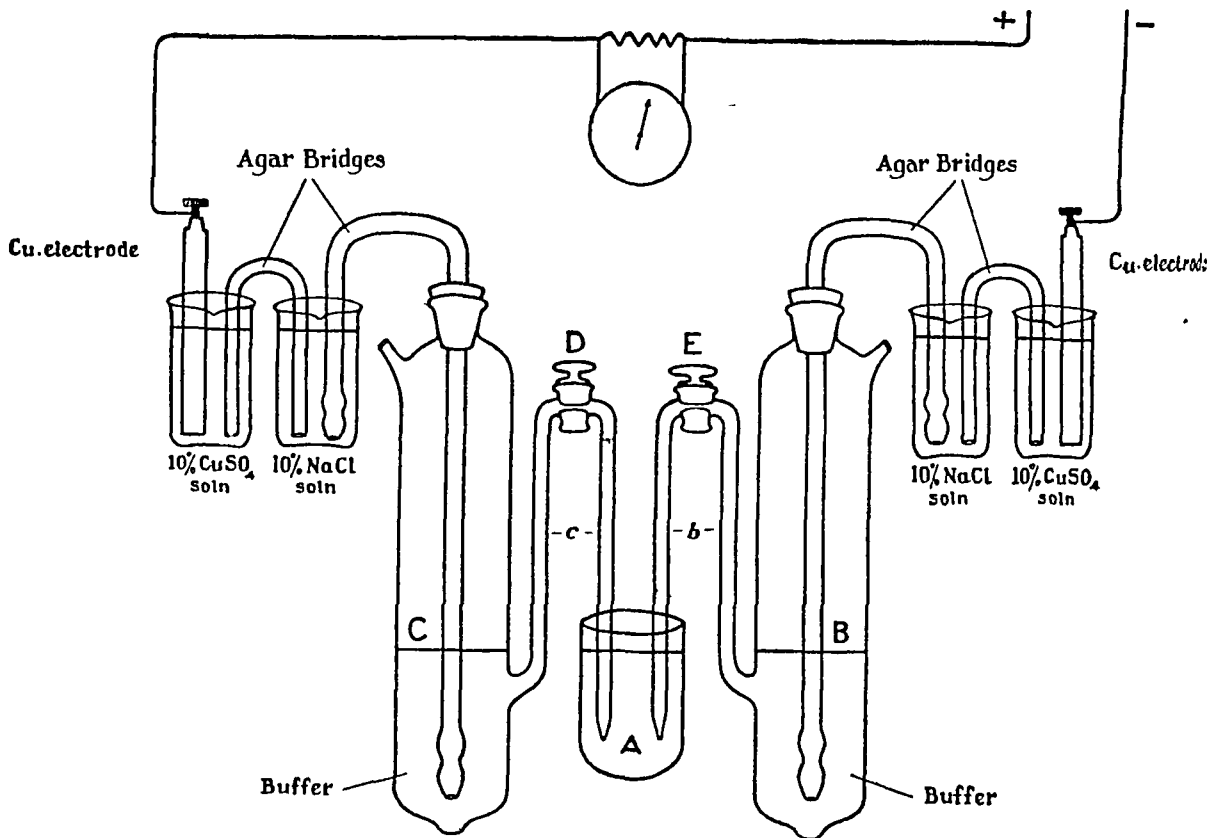


Diagram showing arrangement of apparatus used for electrophoresis.

source of current employed is a 220 volts A. C. street main, converted into 240 D. C. by means of a combined transformer and valve rectifier.

A, B and C are filled with the buffer, care being taken to avoid air bubbles in the side arms of the electrode vessels. The solutions in the three vessels are brought to the same level by opening the taps in the side arms. The taps are then closed and a known amount of buffer is carefully removed from A and replaced by exactly the same volume of ascorbic acid solution in the buffer. The volume of buffer removed from A and that of buffer containing ascorbic acid added must be identical; otherwise siphoning of the ascorbic acid from the central chamber into either electrode vessel may take place.

After running in the current for about 24 hours, taps D and E are closed, and the electrode vessels taken out. A few drops of the liquid from the tips of either electrode vessel are run out into the central chamber, and the outside of the side arms that were dipping into the central chamber washed with distilled water into the central chamber. The contents of A, B and C are then made up to volume, and aliquots titrated against 2:6 dichlorophenol-indophenol, the procedure adopted being that previously used in the laboratory for chemical estimation of vitamin C (Ranganathan, 1935).

The buffers used ranged from pH 1.146 to pH 3.679 and pH 8.575 to pH 12.972, made up of glycoll and either hydrochloric acid or sodium hydroxide, and pH 5.288 to pH 8.043, made up of a mixture of secondary and primary phosphates, according to the tables given in Clark's 'Determination of H-ions' (1920). Varying amounts of current passed through the ascorbic acid solution in the central chamber, depending upon the conductivity of the buffer, being large in the extreme acid and alkaline ranges. The amounts of ascorbic acid migrating are calculated on a uniform basis of 100 milliampere-hours (current strength in milliamperes  $\times$  duration of passage of current in hours).

The results of experiments are shown in the accompanying Table and in Figures 1, 2 and 3 :—

TABLE.

pH.		Initial amount in mg. in the central chamber.	Amount in mg. left behind in the central chamber after 24 hours.	Amount in mg. migrated to the +ve chamber per 100 m-a-h.	Amount in mg. migrated to the -ve chamber per 100 m-a-h.	Amount in mg. of ascorbic acid destroyed in 24 hours.	Ascorbic acid migrated, calculated as a percentage of total left at the end of each experiment.
1.146	..	30	22.92	1.54	Nil.	3.78	5.9
2.279	..	30	25.85	1.22	..	2.62	4.5
3.679	..	30	21.29	5.67	..	2.15	20.3
5.288	..	30	15.60	6.17	..	10.00	30.9
5.589	..	30	6.86	10.04	..	11.54	52.9
6.468	..	30	8.02	10.13	..	10.37	51.5
7.381	..	30	12.76	10.50	..	11.33	56.3
8.043	..	30	6.30	8.98	..	14.06	56.2
10.140	..	30	2.11	6.75	..	19.13	62.0
11.067	..	30	5.20	6.70	..	16.39	49.4
12.095	..	30	0.55	3.26	..	24.46	58.9
12.972	..	30	0.00	0.36	..	29.42	62.0



It is apparent from the above Table that ascorbic acid migrated to the positive pole in every instance, behaving consistently as an electro-negative substance. The amounts which migrated varied with the pH and the rate of destruction of ascorbic acid.

Figure 1 shows that there is a progressive increase in the amount of ascorbic acid migrating to the positive chamber up to a pH of about 7, followed by a steady

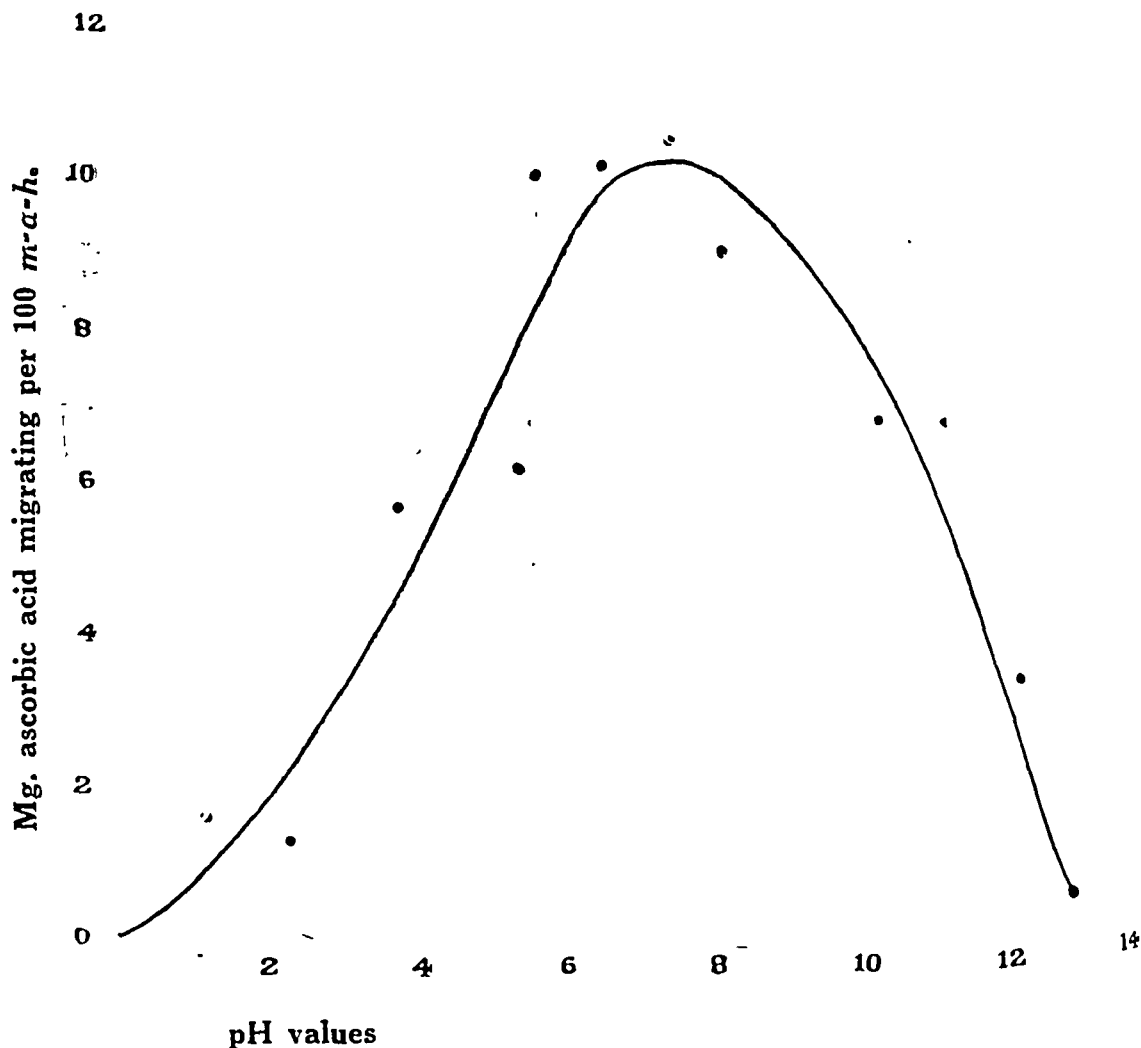


FIGURE 1. Showing the migration of ascorbic acid at various pH levels.

decline in the higher ranges. The shape of the curve might suggest that migration in the alkaline ranges decreases with increase of pH. But this is not so; the smaller amounts migrating are due to the greater destruction of ascorbic acid in the higher alkaline ranges.

In Figure 2, the amounts migrating, calculated as a percentage of the total amount of ascorbic acid present at the end of each experiment, are given. The results show that there is a progressive increase in migration up to a pH of about 7; at ranges above this level, the amount migrating remains stationary.

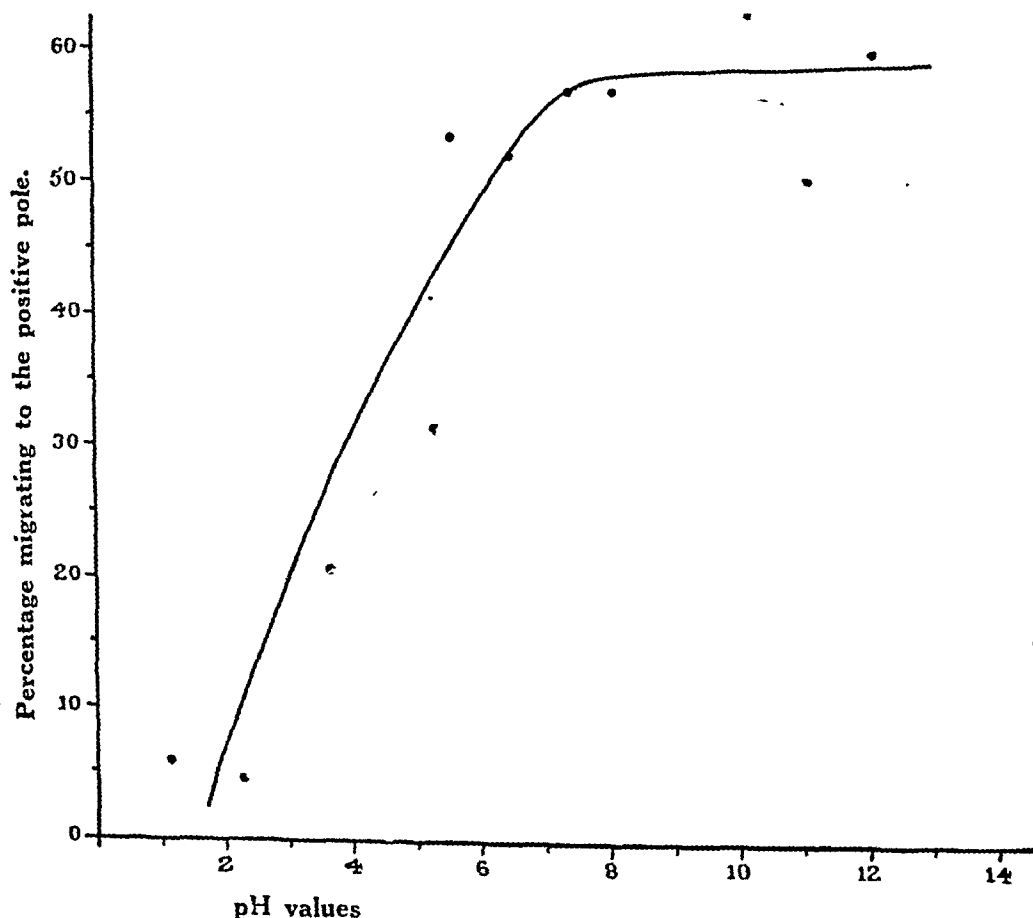


FIGURE 2. Showing the amounts migrating, calculated as a percentage of the total amount of ascorbic acid present at the end of each experiment.

Figure 3 represents graphically the influence of pH on the rate of destruction of ascorbic acid

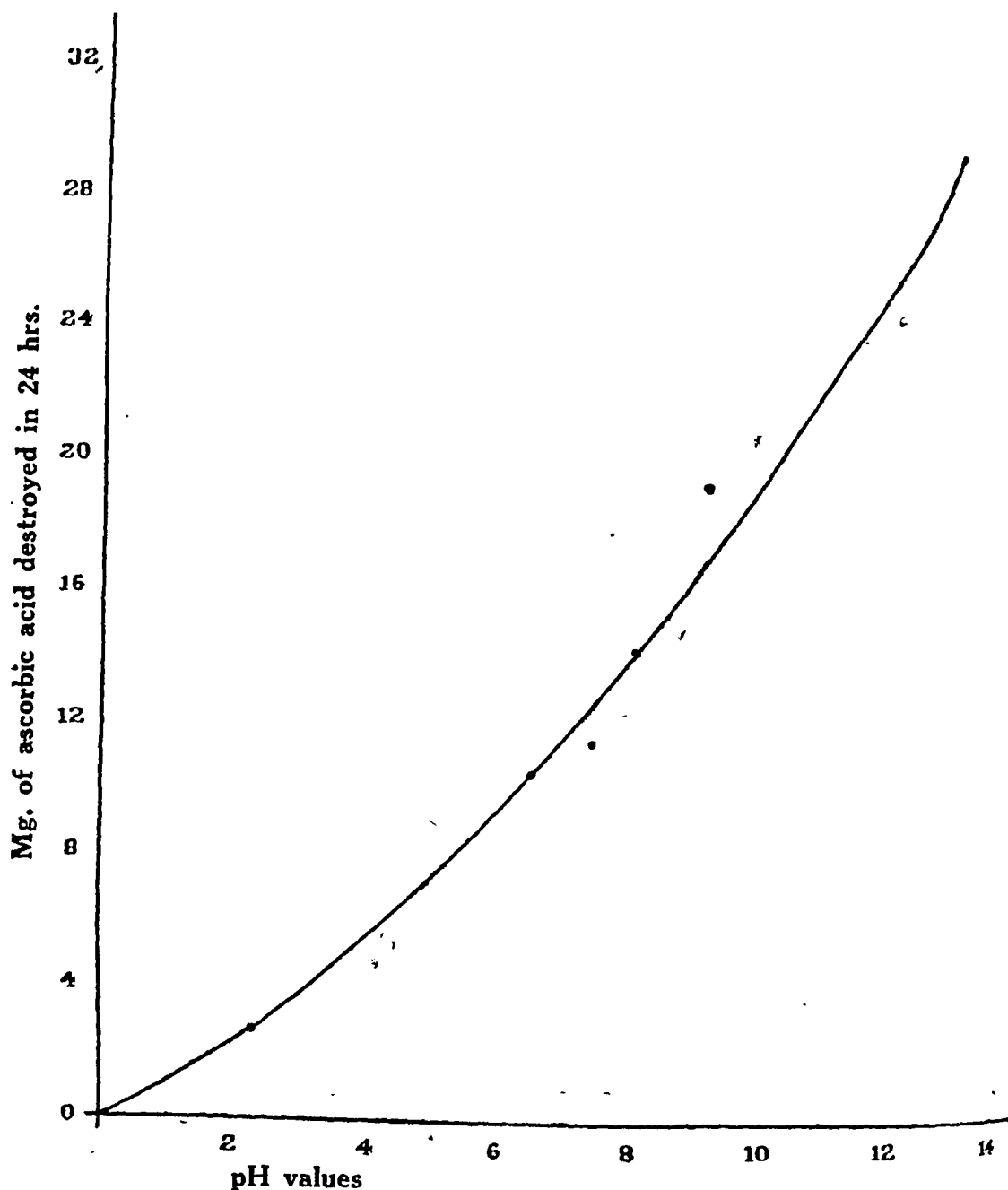


FIGURE 3. Showing the influence of pH on the rate of destruction of ascorbic acid.

#### DISCUSSION.

The evidence so far accumulated tends to show that ascorbic acid is a true acid, having a free carboxyl group, and migrating consistently at all pH ranges like any organic acid. But the idea of a free carboxyl group has been negatived by most

investigators working on the constitution of ascorbic acid, who on the other hand assign it a lactone structure. It seems difficult to explain the electrical migration properties at all pH ranges on the basis of a lactone structure. With a view to ascertaining if any other known lactone would migrate in an electrical field, similar experiments were repeated with phenolphthalein, a lactone, in solution in buffers containing about 20 per cent alcohol. Migration of phenolphthalein could be followed visually in alkaline ranges above pH 8.5, since the substance imparts an intense pink colour to the buffer. There was no migration of phenolphthalein to either side up to a pH of about 8.5, while on the alkaline side of this range it behaved as a true acid, migrating to the positive pole. This observation fits in with the known properties of lactones, viz., formation of an acid in an alkaline media. The results obtained with phenolphthalein seem to suggest that ascorbic acid does not behave like a lactone in solution, as there was migration even in the highly acid ranges and as there was no transition pH at which the lactone was split up, followed by migration to the positive chamber in the higher alkaline ranges, as was observed with phenolphthalein.

Again, Figure 2 clearly suggests that ascorbic acid behaves as a weak organic acid, showing comparatively lower migration figures in the acid ranges, due to the depression of ionization, and stationary migration in the alkaline ranges, indicative of complete ionization.

Herbert *et al.* (1933) have suggested that, though ascorbic acid does not contain a free carboxyl group, its acidic properties are due to an activated  $-\text{CHOH}$  group adjacent to a  $-\text{CO}-$  group, giving a reactive group of the type  $-\text{C}(\text{OH}) : \text{C}(\text{OH})-$ . The question then arises whether organic compounds possessing a similar grouping, a  $-\text{CHOH}$  group adjacent to a  $-\text{CO}-$  group, would migrate in an electrical field. Fructose is known to contain this group and to possess acidic properties as well. To ascertain whether fructose would behave similarly to ascorbic acid in an electrical field, migration experiments were performed with fructose at pHs ranging from 1 to 13. In no instance was fructose found to migrate.

Titration curves as determined by the hydrogen electrode (Birch and Harris, 1933), and by the more refined glass electrode, give concordant results, and show that ascorbic acid in solution is a monocarboxylic acid. The present experiments have afforded evidence for the existence of a free carboxyl group in the molecule of ascorbic acid in solution. Perhaps all or most of the hitherto observed properties of ascorbic acid could be reconciled with this assumption.

#### SUMMARY.

1. The electrical migration of ascorbic acid was studied at various pH levels, ranging from 1.0 to 13.0. It was found to act as an electro-negative substance, migrating always to the positive pole.

2. The electrical migration at all pH levels shows it to be a true acid, with a free carboxyl group, and not a lactone, as phenolphthalein, a lactone, behaves differently in an electrical field.

3. The explanation of the acidic character of ascorbic acid, as being due to a  $-\text{CHOH}-$  group adjacent to a  $-\text{CO}-$  group, is not borne out, since fructose,

possessing a similar group, behaves differently to ascorbic acid in an electrical field.

4. The influence of pH on the rate of destruction of ascorbic acid was also studied.

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## THE CHEMICAL NATURE OF VITAMIN B<sub>1</sub>.

BY

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DURING recent years the problem of the chemical nature of vitamin B<sub>1</sub> has been extensively studied. Investigation of the absorption spectrum in the ultra-violet region (Heyroth and Loofbourow, 1934 ; Peters and Philpot, 1933 ; Smakula, 1934 ; Holiday, 1935) has shown that the molecule includes a pyrimidine structure to which a pyrrole ring is attached by a carbohydrate grouping. Further evidence in support of this structure is provided by the X-ray analyses of Bernal and Crowfoot (1933), whose researches helped to establish the identity of pure vitamin B<sub>1</sub> preparations from various laboratories, and by the chemical work of Windaus *et al.* (1934), Williams *et al.* (1935), Peters (1935), and Kinnersley *et al.* (1935). These latter investigations, purely chemical in nature, converge to show that the vitamin possesses a pyrimidine structure to which a thiozole ring is connected by a pentavalent N atom. Different methods of splitting the molecule were adopted, but there is general agreement that it includes labile groups one of which is an acid (a sulphonic acid). Chemical and physical investigations both suggest that vitamin B<sub>1</sub> belongs to the class of substances known as ampholytes.

A third line of attack, focussed on the ampholyte nature of the molecule, is opened up by physico-chemical methods. If the molecule includes a central basic group, with other groups attached to it, it should be possible, depending on the strength of the binding force, to discover the nature and number of these groups and locate their places in the molecule. Birch and Harris (1935) and Moggridge and Ogston (1935) studied the problem titrimetrically, using hydrogen as well as glass electrodes. The former workers deduced the existence of a basic group, with a pK value 4.9, the molecular weight of which, calculated from the equivalent of alkali used, was 330 ; and also of an acid group, pK 9.0. They point out the presence of a distinctive 'pseudo-acid' arrangement in the molecule. Moggridge

and Ogston obtained similar results except that they postulate a (more weakly) basic group at pK 3.4, and locate the basic group corresponding to that recorded by Birch and Harris at pK 4.8 instead of 4.9. According to these workers the pseudo-acid group is in the region of pH 9.0.

Sankaran and De (1934) investigated the problem by electrophoresis, using an acid clay adsorbate (the international standard preparation) as the source of vitamin B<sub>1</sub>. Their results are not in agreement with those previously recorded, probably because they used the adsorbate instead of the vitamin B<sub>1</sub> itself. Birch and Guha (1931) and Ghosh and Guha (1935) obtained by electrical migration experiments a result more in conformity with that of other workers, showing that vitamin B<sub>1</sub> behaves as a base as far into the alkaline range as pH 8.5. Their study was not extended beyond a pH of 8.5.

The present paper records a re-investigation of the problem by electrophoresis, macro- and micro-, in which both chemical and biological tests for vitamin B<sub>1</sub> were used.

#### PREPARATION OF VITAMIN B<sub>1</sub>.

Crystalline preparations of vitamin B<sub>1</sub> were made in the laboratories from brewer's top yeast, by the procedure described by Kinnersley and his co-workers



FIGURE 1. Photomicrograph  
of vitamin B<sub>1</sub> crystals  
( $\times 120$ ).

(1933, 1935). Their purity was tested by physical, chemical and biological methods. The crystals obtained were transparent with a rectangular plate-like structure (see photomicrograph, Figure 1). The preparations were highly soluble in water and alcohol and showed the characteristic absorption band at  $260m\mu$ . On the addition of diazotized-sulphanilic acid, in the presence of a drop of formaldehyde, a pure pink tint was obtained; this was fairly stable for a period of 20 hours or more. The preparations were tested biologically on pigeons showing head retraction after being fed for 2 to 3 weeks on a diet of highly milled, washed, raw rice. One preparation produced cures in doses of  $2.5\gamma$ , but the minimum curative dose of the preparation used in the experiments was about  $15\gamma$ ; the presence of small quantities of inactive materials, along with the crystals, probably accounts for the fact that this

vitamin B<sub>1</sub> preparation was less potent biologically than others described in the literature.

#### ELECTROPHORESIS EXPERIMENTS.

Aqueous solutions, containing a drop of dilute hydrochloric acid to act as stabilizer, were made so that 1 c.c. of the solution contained 100 $\gamma$  of the vitamin preparation. For each experiment at the various levels of pH, 3 c.c. of the solution (containing 300 $\gamma$ ) were used; to this was added 5 c.c. of either phosphate or glycocoll buffer solution of Sørensen (Clark, 1920). In two experiments at pH 11.0 and 12.0 respectively less concentrated solutions of vitamin B<sub>1</sub> had to be employed, owing to lack of sufficient material.

The electrophoresis apparatus used was that figured in the preceding paper by Ranganathan and Sankaran (page 214).

Vessels A, B and C are filled with the buffer solution to the extent shown in the diagram, the side tubes *c* and *b* being completely full. Chamber A, which is a short length of the bottom portion of a test-tube, is also initially filled with the buffer solution. Communication is established between the three chambers by opening the taps, and the levels equalized. Closing the taps, the vitamin solution is introduced into A, after a corresponding volume of buffer has been removed. The taps are then opened and a current from a D. C. source of 220 volts passed for 6 hours with non-polarizable electrodes.

The pH of the solutions in the various chambers was tested colorimetrically before and after the passage of the current. On the completion of the 6-hour period, the contents of the three chambers were brought to pH 3 and concentrated on a water-bath to a small bulk (5 c.c.). On removing the side tubes from the central chamber, an amount of solution roughly double that contained in the portion of the tubes dipping in the solution was returned to the central chamber; this precaution balances any loss of material from the central chamber which may occur as a result of diffusion or mechanical removal. The major part of each solution was stored in well-corked tubes in a fridaire for subsequent biological testing; the remainder was used for chemical analysis.

#### CHEMICAL ANALYSIS.

Chemical assay of vitamin B<sub>1</sub> depends on the formation of a suitable pink colour with diazotized-sulphanilic acid, the details of the method of investigation being described by Kinnersley *et al.* (1934). The quantity of vitamin B<sub>1</sub> present in test solutions is estimated by comparing the intensity of the pink colour with a mixture of Sørensen's acid and alkaline phosphates (2 and 8 vols. respectively), containing, in 103 c.c., 100 c.c. of phosphate mixture and 3 c.c. of 0.02 per cent phenol red. Equivalent quantities of the buffers were tested to eliminate the possibility of disturbing colour effects being produced by these alone; in no case was any colour observed. In mixtures containing glycocoll and vitamin B<sub>1</sub> a yellow tint in addition to the pink colour characteristic of the vitamin was observed on addition of the reagent. To allow for fading due to the presence of impurities, the intensity of colour was estimated after 30 minutes and again after 20 hours; actually very little fading was observed to take place. The results of the



chemical tests carried out 20 hours after the addition of the reagent are given in Table I :—

TABLE I.

*Migration of vitamin B<sub>1</sub> in an electric field.*

pH.	Original quantity of vitamin B <sub>1</sub> (γ) preparation in central chamber.	Quantity (γ) migrating to positive pole.	Quantity (γ) migrating to negative pole.	Nature of charge.
5.2	300	0.0	75.0	+
8.0	300	0.0	100.0	+
9.2	300	0.0	32.0	+
9.8	300	41.0	21.0	± iso-electric region.
11.0	300	50.0	0.0	—
11.0	200	36.0	0.0	—
12.0	100	25.0	0.0	—
12.9	300	114.0	0.0	—

Strength of current = 2 milliamps, running for 6 hours at a P. D. of 220 volts D. C.

## BIOLOGICAL EXPERIMENTS.

Healthy pigeons, between 210 grammes and 350 grammes in weight, were fed on washed milled raw rice. Within 15 to 28 days the majority developed 'polyneuritis'. Nearly all the birds used for assay showed head retraction in a varying degree.

Test materials were administered through a small catheter inserted in the œsophagus. The disappearance of head retraction within 2 hours indicated a positive test, experience having shown that improvement takes place within this period when an adequate dose of vitamin B<sub>1</sub> is given. Besides the head retraction

many of the birds had paresis of the legs and a considerable number showed green diarrhoea. In 'positive' cases the former usually disappeared concurrently with head retraction, but the diarrhoea often persisted.

Birds showing no improvement after 2 hours usually died within one or two days. Occasionally slight improvement after 2 hours was noted, but this was not maintained and subsequently the birds grew rapidly worse. In these cases a cure was sometimes obtained by re-enforcing the initial test dose. A number of birds which showed no response after 2 hours were given 100 mg. of the international vitamin B<sub>1</sub> standard to test their 'curability'. This produced a disappearance of the head retraction.

The results of these experiments are given in Table II :—

TABLE II.

*Biological testing of solutions obtained by electrophoresis.*

pH.	SOLUTION FROM POSITIVE POLE.			SOLUTION FROM NEGATIVE POLE.		
	Estimated amount of vitamin B <sub>1</sub> in test dose (γ).	Birds treated.	Results.	Estimated amount of vitamin B <sub>1</sub> in test dose (γ).	Birds treated.	Results.
5.2	0.0	2	— —	25.0	3	+
8.0	0.0	2	— —	40.0	2	+
9.0	0.0	2	— —	{ 21.5 10.5	2	+
10.0	14.6	2	+		1	+
11.0	10.0	3	— —	0.0	2	+
11.0	36.0	1	—	0.0	3	—
12.0	{ 12.0 25.0	1	+	0.0	1	—
12.0		1	+	0.0	1	—
12.0	20.0	1	—	0.0	1	—
12.9	40.0	2	— —	0.0	3	+
						—

+ = cure.

— = no cure.

The chemical tests described indicate that vitamin B<sub>1</sub> behaves as an electro-positive substance up to a pH of 9.0. At pH 10.0 migration is bi-polar. The

biological tests in solutions obtained from experiments in the acid range were clear-cut and satisfactory; beyond pH 10.0, however, they do not merit this description. Two negative pole solutions, shown by the chemical test to be devoid of vitamin B<sub>1</sub>, produced 'cures', and on the positive side there was a lack of relation between the quantity of vitamin in the test dose and its curative effect. Failure to obtain 'cures' in certain cases with solutions from the positive pole in the alkaline ranges was probably due to giving insufficient amounts of the vitamin. No explanation can be offered of the two 'cures' produced by negative pole solutions at pH 11.0 and pH 12.9 respectively. In a general way, however, the biological tests confirm the chemical tests.

#### MICRO-CATAPHORESIS.

In order to throw further light on the problem, a series of micro-cataphoresis experiments were carried out.

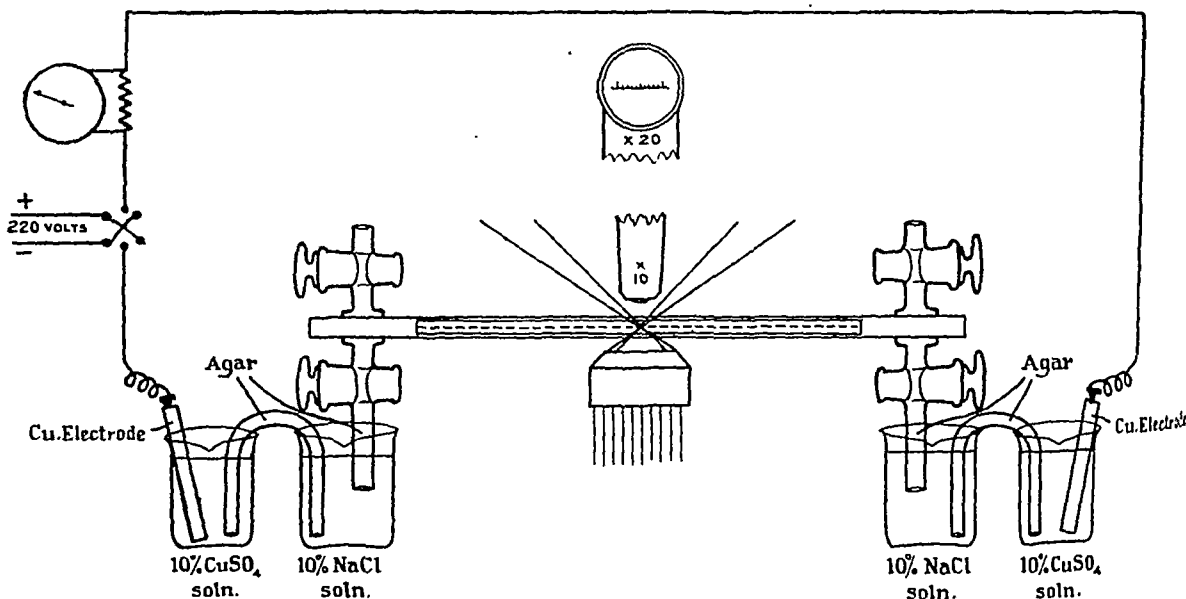


FIGURE 2. Arrangement of apparatus used for micro-cataphoresis experiments.

Abramson (1934) has found that the migration velocity of proteins in an electric field is similar to that of the same proteins in concentrated solution adsorbed on quartz particles; it might be inferred from this that quartz particles have no charge of their own. It was found, however, that a quartz suspension prepared according to the method described by Abramson displayed a negative charge, and that an alteration in the charge of quartz particles resulted from the presence of adsorbed vitamin B<sub>1</sub>. Kieselguhr behaved similarly. In Abramson's work, the strong charge, carried by the proteins which were in high concentration, probably masked the charge of the quartz particles, whereas in the experiments described the vitamin B<sub>1</sub> was present in dilute solution. In the present work the micro-cataphoretic method of Abramson and his collaborators was used, with, however, the following difference in principle: the migration velocity of the carrier with and

without the presence of adsorbed vitamin B<sub>1</sub> was determined, and the difference between the two velocities was attributed to the nature and amount of the charge carried by the vitamin. In place of metallic electrodes used by other workers to lead the current through the solution under investigation (Brown and Broom, 1931, 1936) a non-polarizing arrangement (Figure 2) with an agar block separating the test solution from the electrodes was used. This excludes the possibility of the solution changing its pH as a result of the passage of the current.

#### EXPERIMENTAL.

Kieselguhr, which adsorbs vitamin B<sub>1</sub>, was used in these experiments. It was washed with several changes of distilled water and finally a suitable distilled water suspension was made. One c.c. of the suspension was mixed with 0.5 c.c. of buffer solution and 1.0 c.c. of solution containing 10% of vitamin B<sub>1</sub> crystals. One c.c. of distilled water was added to control solutions in place of the vitamin B<sub>1</sub> solution. The solutions, after standing for 5 minutes, were put in the micro-cataphoretic cell [the cell of Brown and Broom (1931) modified by Millwood] and the migration of particles moving midway between the roof and floor of the cell was observed under darkground illumination. The particular cell used in this work is 16 cm. long, 0.78 mm. deep and 5 mm. wide. The current used was 220 volts D. C., its strength being measured by a galvanometer and shunt included in the circuit. The time taken by a particle, chosen at random, to cover a distance of 1 mm. was determined by means of a micrometer scale in the eye-piece. Velocities were also measured with the current reversed. Table III gives the observed velocities at different levels of pH, each figure representing the mean value of 10 to 20 observations with both directions of current :—

TABLE III.

(A) *Migration velocity of Kieselguhr particles alone.*

pH.	Current strength in m. a.	Average time in seconds taken by Kieselguhr particles <i>without</i> adsorbed B <sub>1</sub> to cover 1 mm.	Calculated time in seconds taken per m. a., per mm.	Migration velocity per 1 m. a. [mm. per sec.].	Nature of charge.
5.9	0.66	2.2	1.45	0.685	Negative.
8.0	1.00	1.7	1.70	0.600	"
8.9	1.33	2.05	2.76	0.362	"
10.0	1.08	3.00	3.24	0.308	"
11.0	1.08	3.00	3.24	0.308	"
12.0	1.16	2.80	3.24	0.308	"

TABLE III—*concl'd.**(B) Migration velocity of Kieselguhr particles with adsorbed vitamin B<sub>1</sub>.*

pH.	Current strength in m. a.	Average time in seconds taken by Kieselguhr particles <i>with</i> adsorbed B <sub>1</sub> to cover 1 mm.	Calculated time in seconds taken per m. a., per mm.	Migration velocity per 1 m. a. [mm. per sec.].	Nature of charge.
5.9	0.83	10.5	8.72	0.114	Negative.
6.0	1.08	2.2	2.38	0.42	„
8.9	1.33	6.2	8.246	0.121	„
10.0	1.00	2.6	2.6	0.384	„
11.0	1.16	2.0	2.32	0.43	„
12.0	1.16	2.3	2.67	0.374	„

In Table IV the differences between the migration velocities of the particles in the presence and absence of the vitamin are calculated :—

TABLE IV.

*Difference in migration velocities of Kieselguhr particles caused by adsorbed vitamin B<sub>1</sub>.*

pH.	Difference.	Nature of charge.
5.9	0.571	Positive.
8.0	0.180	„
8.9	0.241	„
10.0	0.072	Negative.
11.0	0.092	„
12.0	0.048	„

Since the concentration of vitamin at various pH levels was the same, it is assumed that the differences in velocity were due to the charge of the vitamin. In Figure 3 the data given in Table IV are shown graphically :—

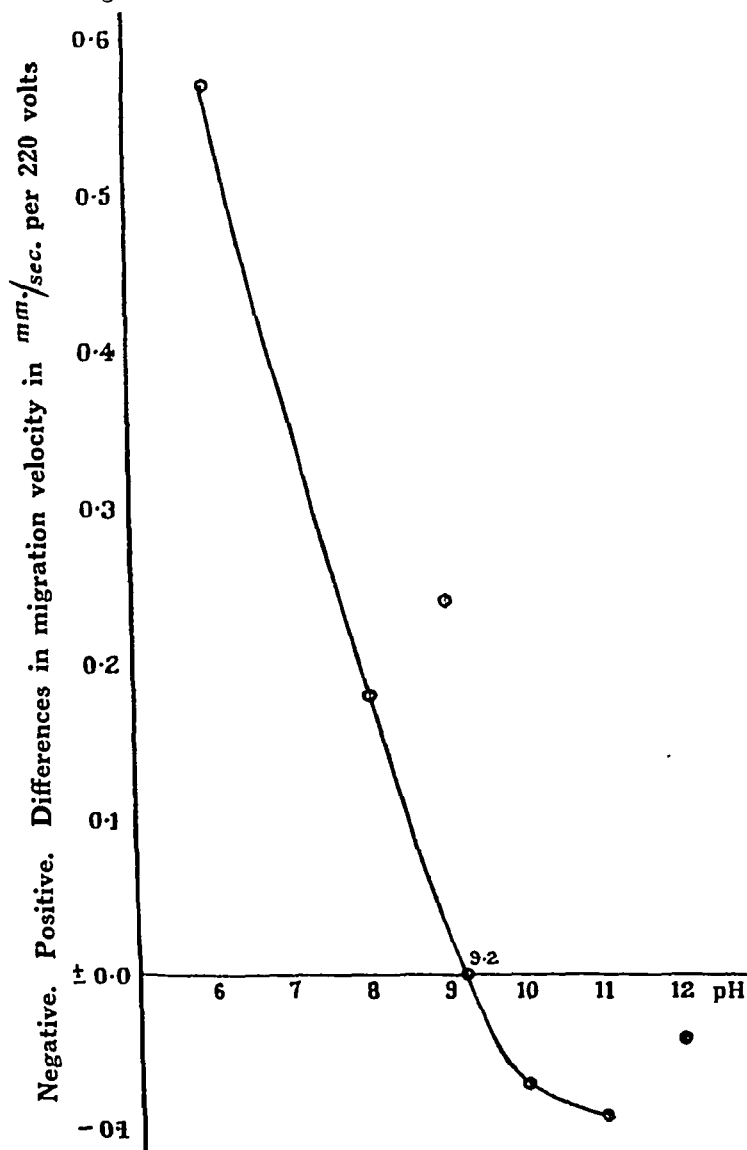


FIGURE 3. Differences in migration velocity of Kieselguhr particles at different levels of pH.

A further indication of the position of the iso-electric point is provided by the current strength carried by the solutions at each level of pH with and without adsorbed vitamin. Theoretically the vitamin possesses minimum potential at the

iso-electric point, and it seems justifiable to assume that the difference between the conductivity of the Kieselguhr suspension alone and that of the suspension containing vitamin B<sub>1</sub> will be least at this point. Galvanometer readings at each hydron level, and the differences between the two sets of readings, are given in Table V. In Figure 4 the difference is displayed graphically. It will be observed that the

TABLE V.

*Strength of current conducted by Kieselguhr suspension with and without adsorbed vitamin B<sub>1</sub>.*

pH.	Galvanometer reading with vitamin (m. a.).	Galvanometer reading without vitamin (m. a.).	Difference (m. a.).
5.9	0.83	0.66	0.17
8.9	1.08	1.00	0.08
8.9	1.33	1.33	0.00
10.0	1.00	1.00	0.00
11.0	1.25	1.08	0.17
12.0	1.25	1.16	0.09

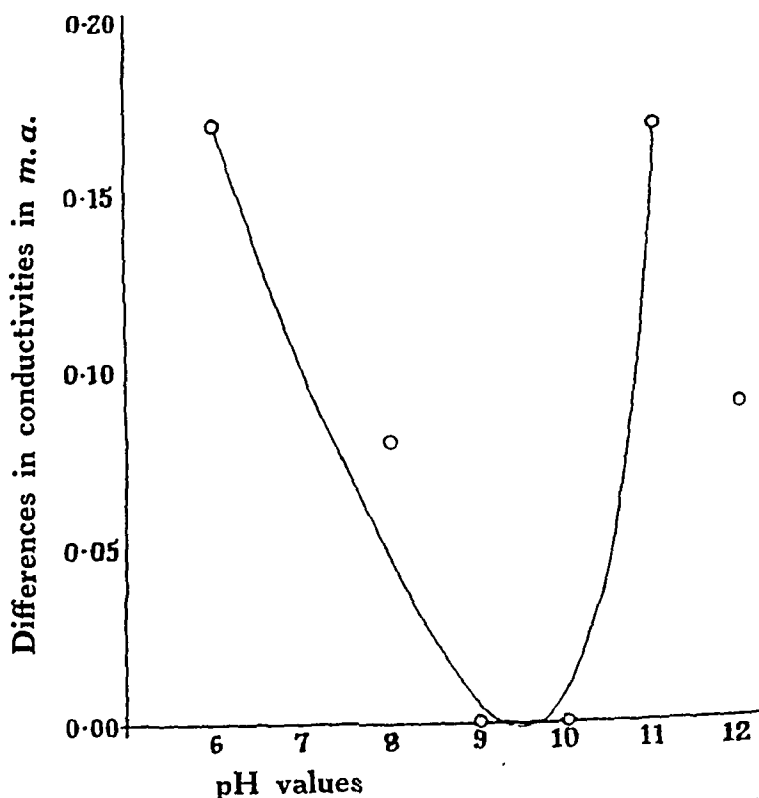


FIGURE 4. Differences in conductivities of solutions at different levels of pH.

difference is minimum between pH 9.0 and pH 10.0 and that this series of observations, while they cannot sharply define the iso-electric point, are in line with the rest.

The results of the micro-cataphoresis experiments suggest that the iso-electric point lies between pH 9.0 and pH 10.0 and nearer pH 9.0 than pH 10.0.

## SUMMARY.

The problem of the iso-electric point of vitamin B<sub>1</sub> was investigated by electrophoresis experiments and an attempt was made to confirm the findings by biological tests. The same problem was also studied by micro-cataphoresis. The experiments described converge to show that the iso-electric point lies between pH 9.0 and pH 10.0, nearer the former than the latter.

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## IMPORTANCE OF PHYTOSTERYL ACETATE TEST IN DETECTING HYDROGENATED FATS IN GHEE.

BY

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THE phytosteryl acetate test is the only reliable test for detection of vegetable fats in ghee and is considered to be the 'final court of appeal' (Bolton and Revis, 1913). The reliability of this test is seriously affected if a small quantity of paraffin wax is added to a mixture of animal and vegetable fats and this fact is taken advantage of by those whose interest is to sophisticate butter or ghee for commercial purposes. The influence of paraffin wax on the melting point of phytosteryl acetate crystals is remarkable. The presence of as small a quantity as 0.02 per cent interferes with the test by lowering the melting point of the crystals much below 100°C. and thus renders the test absolutely useless. Polenske devised (Lewkowitsch, 1921a), however, a method for detecting paraffin in butter and also removing it from the mixture before the actual test is applied.

Lately, another danger in the form of 'vegetable ghee' has appeared on the scene and has already been proved to be a potent factor in upsetting this important test. Vegetable ghee is a mixture of hydrogenated vegetable oils and is extensively used nowadays as an adulterant of ghee and its detection is therefore of utmost importance to the public analysts. During hydrogenation of the vegetable oils at high temperature (250°C.) the phytosterols are completely reduced to resinous hydrocarbons (quoted by Godbole and Sadgopal, 1930) and the characteristic crystals of these sterols are not formed. The hydrocarbons thus produced do not form the sterides with digitonin and on acetylation they produce no acetates. But if the test is properly carried out and every step is carefully watched, certain changes are noticed which furnish a clue for identification of hydrogenated vegetable fats in ghee.

There are two important methods for carrying out this test:—

- (1) the usual saponification and ether extraction method, and
- (2) the digitonin method as described by Klostermann (1913). The second method is very simple and less laborious but more expensive than the first one which takes longer time and sometimes produces troublesome emulsions 'on the slightest provocation' (Elsdon, 1926). But in spite of these drawbacks the saponification method is adopted for detection of hydrogenated fats. The digitonin method is not suitable for this purpose as digitonin combines with only the sterols to form the digitonin sterides and has got no action on the hydrocarbons, the detection of which forms the basis for this investigation. The saponification method as described in some of the standard books is vague and does not help much. A correct method has been described in this paper in detail and if it is followed carefully the result will be quite satisfactory and no emulsion will be formed to spoil the experiment.

(1) About 100 grammes of adulterated ghee are taken in a litre flask and about 200 c.c. (double the amount of ghee) of 2 N alcoholic potash [almost colourless and prepared from KOH (Merck's G. R.) and Merck's absolute alcohol] are added and saponified on a water-bath under a reflux condenser for about three hours. Instead of taking Merck's alcohol, ordinary Indian-made absolute alcohol may be used after purifying it by treatment with KOH and silver nitrate as described by Lewkowitsch (1921b). After saponification the soap is cooled, transferred to a two-litre separator and diluted with about 800 c.c. of water. The level of the soap solution on the separator is marked with a grease pencil. Three hundred c.c. of ether are now added and the separator is very gently tilted up and down for about half a dozen times—shaking at this stage is to be strictly avoided as it results in forming an emulsion. The ether is allowed to separate. If the separation is not fairly quick and a fluffy layer appears just below a thin layer of ether a few drops of alcohol would hasten the separation and about 200 c.c. of ether would be separated—the remaining 100 c.c. being taken up by the soap solution to form a harmless emulsion and is indicated by the pencil mark. The separated ether is transferred to a litre flask. The second extraction with another lot of 300 c.c. of ether is made but this time with a gentle shaking for about half a minute. It is extracted for the third time by shaking gently for about five minutes with 200 c.c. of ether. The fourth extraction by shaking vigorously with 200 c.c. of ether is made but the shaking is continued intermittently for about 15 minutes. If after any of these extractions the ether does not separate and appears to form an emulsion—a crisis which is not likely in this method, may be got over by diluting the soap solution with 200 c.c. to 300 c.c. of water and adding more ether (about 200 c.c.) and tilting up and down as was done in the beginning and the extraction is proceeded with very cautiously avoiding shaking as far as possible and completing the extraction in five or six stages.

(2) The combined ether extracts are distilled and the ether is recovered for subsequent extractions. The residue in the flask consisting of some unsaponified fat, soap, alcohol, water, all the sterols and other unsaponifiable matters including the resinous hydrocarbons, is again saponified with 20 c.c. of the same alcoholic potash under a reflux condenser for about an hour. It is then cooled and diluted

with about 100 c.c. of water and the soap solution is transferred to a smaller separator (about 300 c.c.) and extracted with 100 c.c. of ether with gentle shaking for about two minutes. The ether is separated and transferred to another separator. The extraction is repeated twice with 50 c.c. of ether with gentle shaking and twice more with 50 c.c. of ether with vigorous shaking. The combined ether extracts are washed thrice with about 30 c.c. of water each time. The washed ether containing the sterols and the hydrocarbons with traces of water is filtered through a dry filter-paper into an Erlenmeyer flask and distilled to dryness when the following changes are to be observed in the residue: (a) a light brown mass is obtained with light or deep brown droplets of a sticky resinous hydrocarbon which appears to be the characteristic feature of the presence of hydrogenated fats, (b) these droplets readily dissolve in hot alcohol but reappear on evaporating and cooling, (c) if the amount of hydrogenated fat is small only a sticky, brown substance is found at the bottom of the flask. In some hydrogenated fats, e.g., in Cocogem, the droplets are not deep brown but yellowish in colour resembling oil globules. If the amount of hydrogenated fats is 20 per cent or less, the residue becomes crystalline and the droplets are smaller and fewer. If on the other hand it exceeds 25 per cent the residue becomes amorphous and sticky and the droplets are larger in size and in number.

(3) The residue is now dissolved in about 5 c.c. of absolute alcohol on a water-bath and is transferred to a small glass evaporating basin. The flask is rinsed twice with about 2 c.c. of alcohol and the washings are transferred to the basin. On evaporating, the crystals of cholesterol begin to appear—some floating as a thin film on the surface and some as a white deposit. The following facts are to be noted at this stage: (a) the deposit is not perfectly crystalline and is slightly tinged brown, (b) the mother liquor is also brown and turbid and not clear as in the case of pure ghee, (c) under the microscope the cholesterol crystals will be found but no phytosterol crystals nor the mixed forms (Lewkowitsch, 1921c), (d) a colourless amorphous deposit is found scattered all over the field and in highly adulterated samples, this amorphous deposit forms the main bulk in the field of the microscope and the detection of the cholesterol crystals becomes comparatively difficult, and (e) on evaporating the contents of the basin to dryness, the brown droplets re-appear.

(4) The contents of the basin are now evaporated to dryness on a water-bath and about 3 c.c. (3 c.c. to 5 c.c.) of pure acetic anhydride are added and the basin is covered with a watch-glass and gently heated on a wire gauge over a small flame to boiling and in a few minutes the residue is dissolved and acetylated. If it does not dissolve readily, little more acetic anhydride may be added. The basin is then transferred to a water-bath and heating is continued to expel the excess of anhydride—the watch-glass being removed to hasten evaporation. When there are only a few drops left in the basin, it is cooled and on cooling the sterol acetates set to a brown mass. About 5 c.c. of 95 per cent alcohol are now added. It is warmed and allowed to evaporate and crystallize. The crystals (cholesteryl acetate and also of phytosteryl acetate if present) are filtered through a small filter (diameter of the funnel is about 1") moistened with alcohol and washed twice with 1 c.c. of cold 95 per cent alcohol. The following points are to be noted at this stage: (a) the colour of the mother liquor is distinctly brown, (b) the resinous hydrocarbons are seldom seen as brown droplets at this stage but appear as a sticky, brown coating at the bottom of the basin and cannot be detached from it, this stickiness being

characteristic and easily felt with the finger tip. In pure ghee no such changes take place and the crystals are not sticky and can be easily and completely separated from the basin, (c) if the mother liquor, after separation of the crystals by filtration, is evaporated to dryness and the melting point of the dry residue (a mixture containing some resinous hydrocarbons, normal hydrocarbons of ghee and some cholesteryl acetate) is determined, it will be found to vary from 50°C. to 70°C. or even lower, and (d) in pure ghee this residue is a pale yellow liquid at the room temperature and solidifies on cooling.

(5) A few crystals from the filter (the first crop) are taken out with the point of a knife and dried on a hot porous plate and its melting point is determined. The remaining portion of the crystals is transferred completely from the filter to the basin and dissolved again in 5 c.c. of 95 per cent alcohol and allowed to crystallize. The crystals (the second crop) are filtered and washed as before and some of them are taken out and dried for determination of the melting point. By repeating this process five crops of crystals can be easily isolated and their melting point determined. The melting point of the first crop of crystals is about 113°C. in cases of pure ghee but in samples containing hydrogenated fats it may come down to 108°C. (usually 110°C.). The lowering of melting point of the first and possibly of the second crop of crystals is due to presence of the hydrocarbons of low melting point formed during hydrogenation and is therefore of importance for detection of hydrogenated fats in ghee.

In cases of adulteration of ghee with ordinary vegetable fats the steady increase in the melting point of the successive crops of crystals conclusively prove the presence of vegetable oils and fats. But if it is adulterated with hydrogenated vegetable fats no such increase in the melting point is observed—practically the same melting point in every crop of crystals (from the third to the fifth) is obtained, indicating the presence of only the cholesterol of ghee. The appearance of a brown, sticky and resinous substance at the various stages of the experiment is therefore an unmistakable proof of the presence of hydrogenated fats. If the experiment is performed strictly according to the method described above, the presence of 10 per cent hydrogenated fats will be detected without much difficulty. In order to be thoroughly conversant with this method it is advisable to experiment with one or two samples of pure ghee and pure vegetable oils (unhydrogenated) before the actual experiment is performed with adulterated samples, otherwise the phenomena characteristic of hydrogenated fats are likely to be missed. Experiments with Cocogem and two different brands of vegetable ghee (made in Holland) mixed with pure ghee in different proportions varying from 10 per cent to 66 per cent indicated the presence of the hydrogenated fats in all the mixtures. It is stated that hydrogenated fish oils are now imported from Japan for use as an adulterant of ghee. As a genuine sample of hydrogenated fish oil was not available and could not be analysed we are not in a position to state the actual changes that are likely to be met with at different stages of the experiment but we are sure that this test will also detect its presence in ghee because the cholesterol of fish oil is more easily reduced to resinous hydrocarbons even at a lower temperature. It may, therefore, be said that the phytosteryl acetate test still holds the ground and is equally useful for detection of all forms of hydrogenated fats and oils in ghee.

It may be mentioned in this connection that the quantity of ghee (100 grammes) usually recommended for the phytosteryl acetate test is one of the reasons which

stand in the way of detecting vegetable fats and oils in ghee by this test as the formal samples generally received by the public analysts contain about 50 grammes or even less. But a little experience in the technique described in this paper will enable them to take up and successfully analyse 25 grammes of moderately adulterated ghee and give a definite opinion one way or the other.

#### SUMMARY.

The technique of the phylosteryl acetate test has been described in detail. The following indications are important for detection of hydrogenated fats: (1) Formation of brown droplets of a resinous substance on evaporating and cooling the hot alcoholic solution of the resaponified sample or its ether extract. (2) Under the microscope the typical cholesterol crystals partially covered with an amorphous deposit are seen but no phytosterol crystals nor the intermediate forms. (3) On acetylation of the ether extract (sterols and other unsaponifiable matters) the resinous substance forms a sticky, brown coating which cannot be detached from the basin. (4) After acetylation, the residue (sterol acetate and hydrocarbons) is allowed to crystallize from alcohol and five different crops of crystals are obtained. The melting point of the first fraction and possibly of the second is about 110°C. and that of the last three fractions is constant at about 114°C. which is the melting point of cholesteryl acetate. (5) The first filtrate is evaporated to dryness and the residue gives a low and indefinite melting point—varying from 50°C. to 70°C., while in pure ghee the residue is a pale yellow, oily liquid at room temperature and the droplets of the brown and sticky hydrocarbons are not noticed. The digitonin method is quite satisfactory for detection of ordinary vegetable fats in ghee but not so for hydrogenated fats.

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## SOME OBSERVATIONS ON THE INDOPHENOL (2 : 6 DICHLOROPHENOL-INDOPHENOL) REDUCING PROPERTIES OF URINE.

BY

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SINCE the introduction of the method for the titration of vitamin C against 'red ox' dye (indophenol) by Tillmans in 1932, the method has been used by a number of workers. Its application has led to the discovery of the presence of this vitamin in fairly high concentrations in a number of unexpected sites in the animal body, such as the medulla and the cortex of the suprarenal gland, the aqueous and vitreous humours, and the wall of the gut and also the urine. But the evidence put forward to establish the identity of such substance with ascorbic acid can not be said to be quite convincing, at least so far as the urine of normal individuals and other body fluids and tissues are concerned.

Birch and Dann (1934) applied the silver nitrate staining test to a number of tissues which have been found by indophenol titration to contain ascorbic acid, with results which were not always in harmony with those obtained by the latter method. Some tissues found by the titration method to contain ascorbic acid, gave little or no staining with silver nitrate but they thought that their results supported the conclusion formulated by Harris and Roy (1933), viz., 'that a positive result with this test indicated the presence of ascorbic acid but a negative result did not necessarily mean that ascorbic acid was absent'. But this leaves the question as to the criterion for testing the presence or absence of ascorbic acid still unsettled.

Biological tests (the recovery test of Harris *et al.*, 1932) carried out by Birch and Dann to verify the results obtained by the titration method with the eye lens and aqueous humour of the ox (which were found to have appreciable indophenol reducing properties) were unsuccessful and gave no indication of the presence of ascorbic acid. According to these authors the failure of the test suggested not so



much the absence of ascorbic acid in the lens, as the presence of some other substance exerting a positive toxic effect. Another biological test was however tried, with alterations considered likely to diminish the effect of toxicity of the lens, viz., the tooth structure method of Højer as modified by Key and Elphick (1931) and by this method, according to them, undoubted evidence of the presence of ascorbic acid in the lens of the ox's eye was obtained.

In these experiments three of the four animals receiving a supplement of the lens died before the first of the negative control animals, the average time between the commencement of the experimental period and death being  $4\frac{1}{2}$  days for those receiving the dried lens and  $6\frac{1}{2}$  days for the negative controls. The animals receiving aqueous humour lived through the experimental period of nine days but did not show any increase of weight as did the positive controls receiving 3 c.c. of orange juice daily. It should be noted that the detoxicated specimens of the lens were not tested by the curative method.

It is difficult to see how the same specimens administered in the same way (viz., by the mouth) could produce different results with different methods of testing and moreover how it could produce one effect of vitamin C (viz., protection against scurvy) and not the other (viz., increase in weight).

As to the question of the presence and amount of this vitamin in the urine also, conflicting results are reported. Van der Walle (1922) reported that a daily dose of 20 c.c. of fresh normal urine afforded no significant protection from scurvy to guinea-pigs on a scorbutic diet. Harris and Roy (1935) suggested an explanation for this failure on the ground that either the urine was not fed in a sufficiently large dose or it was not fed soon enough after it had been passed, to guard against inactivation of the urine.

Van Eekelen and his co-workers (1933) showed that the indophenol reducing capacity of human urine increased after the consumption of much fruit or of high doses of decitrated lemon juice.

Harris, Roy and Ward (1933) showed that in a number of individuals, the daily output of vitamin C, as measured by the indophenol titration, was surprisingly constant, generally in the neighbourhood of 30 mg. to 33 mg. per day. They also showed that if a normal individual is given a single very large dose of vitamin C, the urinary output of ascorbic acid (indophenol titration) sharply rises, reaching a maximum in about three hours, it then rapidly drops again and within a day or so, reached the normal resting level of about 33 mg. per day, where it remained remarkably steady although the subject may be restricted for a week or more to a vitamin-C free diet. From the results obtained by these workers it is evident that the 'normal' daily loss of about 33 mg. is somewhat greater than the reputed minimum daily requirement for man (viz., 1 oz. of orange or lemon juice containing about 20 mg. of ascorbic acid).

It is somewhat surprising that the system should be so wasteful regarding a substance the absence or diminution of which would involve it in serious complications. In the ordinary course of things, the body excretes those substances, the presence of which in excessive amount would be deleterious to it but no cases of hypervitaminosis C are recorded.

These workers further showed that the urinary output depended both on the immediate vitamin intake and also on the past nutritional history or in other words.

on the state of vitamin saturation and they have suggested the application of this titration technique as a diagnostic test for detecting states of vitamin-C sub-nutrition in human beings.

Harris and Roy (1935) adduce as a proof for the specificity of the titration method the following: the virtual disappearance of the titre in scurvy and its restoration when vitamin C is fed and the proportionality between the titre and the amount of vitamin C in the diet. But from the results obtained by us, to be described later, it will appear that the indophenol titre of the urine is much reduced in other conditions also (*viz.*, epidemic dropsy), without any apparent manifestation of the symptoms of scurvy. Hess and Benjamin (1934) on the other hand have concluded from their experiments on children that under ordinary dietary conditions, the amount of vitamin C present in human urine is so small as to be considered negligible.

Recently, however, Johnson and Zilva (1934) by the daily administration of urine containing 0.5 mg. ascorbic acid (titration method) to guinea-pigs which had subsisted for 14 days on a scorbutic diet found that the anti-scorbutic potency of the urine dose was of the same order as that of 0.5 mg. of ascorbic acid. It must, however, be noted in this connection that the urines used for these biological tests were those of persons who were dosed with large amounts of ascorbic acid (875 mg. and 1,000 mg.) before the urine was collected. These experiments do not prove the excretion of vitamin C through the urine of normal individuals under ordinary dietary conditions.

In the same paper the following observations of these workers are also worthy of note. 'Three of the four experimental subjects while using ordinary mixed diet on which they usually subsist, passed, during the winter months, urine, which for days scarcely reduced indophenol. Even when the titrations were significant, the output of ascorbic acid per 24 hours was of the order of 10 mg. to 15 mg. During the summer the indophenol reducing capacity rose to some extent. The fourth subject used a diet in which bread and potatoes were replaced by green vegetables and in addition he consumed regularly two oranges a day. In consequence his normal urinary output of ascorbic acid was 80 mg. to 150 mg. per day even during the winter. At no time in the period of 8 months, during which the experiments were carried out, were there any indications even of latent scurvy in any of the four subjects'. This is in accord with our own findings. One of us regularly excreted urine, the reducing properties of which were very small yet no apparent manifestation of the symptoms of scurvy could be detected.

The main points which emerge from all these observations are:—

1. Some of the tissues and body fluids have the property of reducing indophenol to an appreciable extent.
2. That in normal human urine, a substance is excreted in variable amounts which has this property.
3. This substance reduces indophenol in the same manner as ascorbic acid.
4. If a person is given a large dose of ascorbic acid containing substances, the indophenol reducing property of the urine is considerably increased for some time.
5. The biological tests give contradictory results and cannot be said to be entirely satisfactory.

So that it appears that the proof of the existence of ascorbic acid in the urine and in other tissue fluids excepting perhaps suprarenal cortex has not been established beyond the possibility of doubt. 2:6 dichlorophenol-indophenol is one of the indicator dyes, and it is very readily reduced. In fact it almost tops the list of such dyes in this respect. It is, therefore, quite conceivable that some other substances present in the urine and tissues are responsible for the reduction of the dye.

So far as the actual method of titration is concerned it is not always possible to titrate the urine immediately after it is passed. This difficulty is especially felt with respect to the hospital patients and with night specimens. Therefore it is necessary that a suitable preservative should be used which will keep the indophenol titre unchanged for at least 18 to 20 hours. The addition of 5 per cent trichloroacetic acid was first recommended. Later on, Harris and Roy (1935) preferred the addition of 10 per cent of glacial acetic acid and remarked that under such conditions the vitamin C in the urine may be preserved for about 10 to 12 hours with relatively little loss. Again, Johnson and Zilva (*loc. cit.*) used sulphuric acid as a preservative. They collected the urine over sulphuric acid (strength or quantity not mentioned). Under these conditions the ascorbic acid was found by them to be stable for several days. Titrations were carried on at pH 3. Acetic acid in their opinion had a less stabilizing effect.

It was, therefore, our object to determine if there was any difference in the titre under the above conditions of titration and under which of the above conditions the urine is preserved more efficiently. Because a part at least of the urine passed during 24 hours must necessarily be preserved for some hours, and, if the preservative is not efficient, that would make the titration values obtained with regard to the 24 hours' specimen relatively of little consequence.

In our experiments urines were usually collected 4 hours after the midday meal.

TABLE I.

Nature of the specimen.	REDUCING PROPERTIES CALCULATED IN TERMS OF MG. OF ASCORBIC ACID PER 100 C.C.			
	TRICHLOROACETIC ACID 5 PER CENT.		GLACIAL ACETIC ACID 10 PER CENT.	
	Immediate.	3 hours after.	Immediate.	3 hours after.
1. Normal (A. C. R.) .. ..	6.6	6.25	5.0	3.4
2. do. " .. ..	6.25	..	5.4	..
3. do. " .. ..	2.85	1.8	2.2	1.2
4. A portion of the above specimen was kept without the addition of either CCl <sub>3</sub> COOH or glacial acetic acid and then titrated after 3 hours as usual.	..	2.5	..	1.4
5. Normal (A. C. R.) .. ..	4.5	4.1	3.40	1.80
6. do. " .. ..	5.0	..	3.40	..
7. do. " .. ..	5.0	..	3.2	..

TABLE I—concl'd.

Nature of the specimen.	REDUCING PROPERTIES CALCULATED IN TERMS OF MG. OF ASCORBIC ACID PER 100 C.C.			
	TRICHLORACETIC ACID 5 PER CENT.		GLACIAL ACETIC ACID 10 PER CENT.	
	Immediate.	3 hours after.	Immediate.	3 hours after.
8. Normal (A. C. R.) .. ..	5.0	..	3.5	..
9. do. (B. C. D.) .. ..	4.0	..	3.0	..
10. A portion of the above urine was boiled for 5 minutes, cooled, made up to original volume and then 10 per cent glacial acetic acid added.	..	..	2.4	..
11. Normal (B. C. D.) .. ..	4.0	4.0	2.8	2.0
12. do. (A. C. R.) .. ..	2.0	2.0	..	..
13. do. „ .. ..	3.7	3.7	..	..
14. Suffering from acute abdominal pain (H. C. M.).	2.0	..	1.25	..
15. Polyarthritis; takes moderate amount of meat every day.	5.4	..	4.4	..
16. do. .. ..	6.2	..	4.8	..
17. Gout (before atophan) .. ..	7.7	5.9	7.0	2.0
18. do. after 9 tablets of atophan ..	6.2	..	3.3	..
19. Cured E. D.; takes moderate amount of meat, brown bread and fruits.	14.2	..	16.0	..
20. Cured E. D. .. ..	16.6	16.6	18.5	13.8
21. E. D. cured .. ..	14.2	10.0 (5 hours).	12.5	3.3 (5 hours).

E. D. = Epidemic dropsy case.

TABLE II.

Nature of specimen.	REDUCING PROPERTIES IN TERMS OF MG. OF ASCORBIC ACID PER 100 C.C.								
	TRICHLORACETIC ACID 5 PER CENT.			ACETIC ACID 10 PER CENT.			H <sub>2</sub> SO <sub>4</sub> (pH 3).		
	Immediate.	3 hours after.	21 hours after.	Immediate.	3 hours after.	21 hours after.	Immediate.	3 hours after.	21 hours after.
1. Normal (A. C. R.)	5.7	5.2	5.5	4.1	3.7	2.8	4.3	3.8	3.25
2. do. „ ..	6.6	..	6.0	4.5	..	2.8	4.5	..	4.10
3. Normal (K. S. M.)	7.2	..	6.6 (5.5) 3 weeks after.	4.4	..	3.2 (1 mg.) 3 weeks after.	4.1	..	3.7 (1.0) after 3 weeks.

From Tables I and II it would appear that :—

1. Even normal individuals excrete variable amounts of the reducing substances through the urine.
2. Their concentration in the urine depends considerably on the diet.
3. The titres by the acetic acid method yield consistently lower values than those obtained by the trichloroacetic method.
4. None of the substances used can be said to be an efficient preservative to guard against inactivation due to storage but if we are to choose,  $\text{CCl}_3\text{COOH}$  seems to be more effective than the others in this respect.

To see whether there is any difference in the titre when different acids of varying concentrations were used, the tests shown in Table III were carried out. For each set, the same specimen of urine was used :—

TABLE III.

Nature of specimen.	$\text{CCl}_3\text{COOH}$ 5 per cent.	IN TERMS OF MG. OF ASCORBIC ACID PER 100 C.C.; 1 C.C. OF THE ACIDS ADDED TO THE INDICATOR.					
		10 per cent acetic acid.	Glacial acetic acid.	50 per cent $\text{H}_2\text{SO}_4$ .	Con. HCl.	HCl. 1 in 4.	20 per cent $\text{CCl}_3\text{COOH}$ .
Normal (A. C. R.)	5.0	3.3	3.3	10.0	40.0	8.0	8.0
0.5 c.c. of 2N acids added to the indicator.							
	$\text{CCl}_3\text{COOH}$ 5 per cent.	10 per cent acetic acid.	Acetic acid.	Oxalic acid.	HCl.	$\text{H}_2\text{SO}_4$ .	
do. „ ..	5.0	3.4	3.0	4.5	6.6	6.6	

These results show that the titre varies considerably with the nature and concentration of the acids.

Some time ago Ahmed (unpublished) made an important observation that the indophenol reducing properties of urine were increased on a high protein diet and that the nitrogen content of the urine and its indophenol reducing capacity ran almost parallel. This observation also does not quite fit in with the assumption that the indophenol reducing properties of normal urine is entirely due to the presence of ascorbic acid. If the whole of the indophenol reducing properties is attributed to the presence of ascorbic acid it is difficult to understand why it should have any bearing upon the protein intake and metabolism.

On the bases of these observations we made some experiments with a view to see if there was any relation between the indophenol reducing properties of the urine and purine metabolism. Uric acid content and the reducing capacity of the urine were determined simultaneously in a number of normal as well as pathological cases, with the following results :—

TABLE IV.

Nature of specimen.	Reducing property in terms of ascorbic acid mg. in 100 c.c.	Uric acid in grammes per litre.	REMARKS.
1. Normal (S. D.) ..	2.5	..	..
2. do. (A. C. R.) ..	4.0	..	..
3. do. (S. D.) ..	2.66	..	..
4. do. (H. M.) ..	2.90	0.32	..
5. do. (A. C. R.) ..	5.3	0.53	..
6. do. (S. D.) ..	less than 0.4	0.16	..
7. do. (P. B.) ..	3.0	0.44	..
8. do. (A. C. R.) ..	5.0	0.47	..
9. do. „ ..	3.0	0.44	..
10. do. (S. D.) ..	1.6	0.26	..
11. do. „ ..	1.4	0.22	..
12. do. „ ..	1.6	0.33	..
13. do. (P. B.) ..	2.3	0.34	..
14. do. (S. L.) ..	2.2	0.32	..
15. do. (N. P.) ..	3.1	0.42	..
16. do. (A. C. R.) ..	2.9	0.42	..
17. do. (S. D.) ..	2.0	0.16	..
18. do. (A. C. R.) {	3.7	0.33	Immediate.
	3.7	0.33	After 4 hours.
19. do. „ ..	2.9	0.32	..
20. do. (H. M.) ..	4.4	0.44	..
21. do. (A. C. R.) ..	4.5	0.45	..

TABLE IV—*contd.*

Nature of specimen.	Reducing property in terms of ascorbic acid mg. in 100 c.c.	Uric acid in grammes per litre.	REMARKS.
22. Normal (S. D.) ..	5.0	0.44	This individual is not a habitual meat or fish eater and in previous cases his urine gave low values but on inquiry it was found that he took fairly large quantities of fish on that as well as the previous day.
23. do. (A. C. R.) ..	5.0	0.53	..
24. do. „ ..	5.0	0.47	..
25. Polyarthritis ..	6.2	0.59	..
26. Gout (before atophan)	7.7	0.64	..
27. „ (after atophan)	6.2	0.48	..
28. Cured E. D.; takes plenty of red atta, meat and fruits.	14.2	0.70	..
29. do. ..	16.6	0.64	..
30. do. ..	14.2	0.50	..
31. Epidemic dropsy ..	1.0	..	..
32. do. ..	1.54	..	..
33. do. ..	1.32	..	..
34. do. ..	1.32	..	..
35. do. ..	1.10	..	..
36. do. ..	2.3	0.35	..
37. do. ..	0.9	0.14	..
38. do. ..	1.1	0.21	..
39. do. ..	0.8	..	..
40. do. ..	1.6	..	..
41. do. (convalescent).	6.0	0.54	..
42. do. ..	0.8	0.21	..

TABLE IV—*concl'd.*

Nature of specimen.	Reducing property in terms of ascorbic acid mg. in 100 c.c.	Uric acid in grammes per litre.	REMARKS.
43. Epidemic dropsy ..	0.8	0.21	..
44. do. ..	1.4	0.26	..
45. do. ..	2.0	0.33	..
46. do. ..	3.2	0.42	..
47. do. ..	0.8	0.21	..
48. do. ..	1.4	0.26	..
49. do. ..	2.0	0.33	..
50. do. ..	3.2	0.42	..
51. do. ..	2.0	0.32	..
52. do. ..	2.1	0.38	..
53. do. ..	2.1	0.33	..

These results indicate that :—

1. There is an undoubted relation between the indophenol titre and the uric acid content of the urine and that there is even a rough proportion between the two in the majority of cases.

2. The excretion of the reducing substances as also of uric acid is low in the urine of persons suffering from epidemic dropsy.

3. That the indophenol titre and the uric acid content increase considerably after the epidemic dropsy patients have been put on for some time to high protein and fruit diets and when all active symptoms of the disease have disappeared.

4. The urine of one individual (S. D.) had usually very little reducing action but on a particular day the titre was found to increase considerably and it was found that on that day and the previous night he had liberal quantities of fish.

5. In one case simultaneous determination of the indophenol titre and uric acid content were made immediately after the urine was passed and, also after 4 hours to see if there was any simultaneous change of the uric acid content with the decrease of the titre value due to keeping, but in this case both sets of values were the same.

Three individuals were put on to a diet containing a large amount of fat (ketogenic diet—240 grammes of fat in 24 hours). After three or four days, acetone bodies were found in the urine in appreciable quantities. The indophenol titres of these specimens were low, showing that a diet rich in fat does not increase the reducing properties of the urine.



We had also the opportunity of testing a few specimens of aqueous humour of the eye and found that they readily reduced indophenol.

1. 12.5 mg. per 100 c.c.

2. 6.6 mg. „ „ „

But these are glaucoma fluids from epidemic dropsy cases kindly supplied to us by Lieut.-Colonel E. O'G. Kirwan, I.M.S., Professor of Ophthalmic Surgery, Medical College, Calcutta, and may have lower reducing property than normal specimens. We were not fortunate enough to secure aqueous humour of the eye from normal individuals and therefore comparisons could not be made.

#### SUMMARY AND CONCLUSIONS.

1. The evidence put forward to establish the identity of the indophenol reducing substance in the urine of normal individuals and in various other tissues, with vitamin C is not conclusive.

2. The biological tests give conflicting results and are unsatisfactory.

3. The titre varies considerably according to the nature and strength of the acids used for titration.

4. Trichloroacetic acid, acetic acid and sulphuric acid are none of them efficient preservatives for urine, but 5 per cent trichloroacetic acid seems to be better than the other two in this respect.

5. With 10 per cent acetic acid consistent lower values are obtained than with trichloroacetic acid.

6. The indophenol reducing property of the urine of individuals suffering from conditions other than scurvy (viz., epidemic dropsy) and even of some normal individuals is usually found to be very low but no apparent symptoms of scurvy are manifest in these cases.

7. There is a significant relation between this reducing property and the uric acid excretion, and the results run almost parallel.

8. The indophenol titre of the urine is not increased on a ketogenic diet.

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## PHARMACOLOGICAL ACTION OF CAMPHOR AND ITS DERIVATIVES.

BY

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### INTRODUCTION.

CAMPHOR has been used in Chinese medicine for many centuries and has also been employed in Western therapeutics during recent years. It is a favourite remedy in almost every Indian home for a variety of ailments. The reputation of camphor as a circulatory stimulant has been widely believed, but recently there has been difference of opinion among the laboratory and clinical workers regarding its pharmacological action and therapeutic uses. Camphor has been claimed to act rapidly as a cardiac stimulant by Gorman (1920). The claim for such action in the face of slow absorption and conversion into an inert compound with glycuronic acid is difficult to explain. It has been suggested that the stimulant action may be merely a reflex action as in the case of alcohol or ether administered either by mouth or subcutaneously. The experimental work done on animals has given variable results regarding its cardiac stimulant properties. While reviewing the literature on the action of camphor on the heart of a frog, Heathcote (1923) pointed out that some observers have found it to be a cardiac depressant, others as a cardiac stimulant and still others believe that it has no action on the normal heart at all. It only stimulates the heart that has been depressed artificially by toxic drugs. By his own observations on the hearts of frogs, rabbits and cats, he showed that there is no convincing pharmacological evidence in support of the view that camphor possesses any value as a cardiac or a circulatory stimulant. Cushney declared that there is no evidence to show that camphor even in large therapeutic doses has any effect except to cause a slight stimulation and this is probably due to reflex dilatation of the skin vessels. In the frog it causes a descending paralysis. Nakagawa (1923) believed that camphor affects the origin and conduction of cardiac impulses and in large doses stimulates

the latent automatism. The heart is slowed down by camphor and its derivatives both in man and in animals. Camphor has been reported to dilate the coronary vessels, but it is not certain that this occurs with therapeutic doses. Sanders (1929) reported that camphor diminished the force of contraction in dog's heart which was regularly stimulated with electric current.

Clinically camphor has been used as a rapid and effective cardiac restorative when the heart is failing or when death is impending. Efforts have been frequently made to determine how camphor acts as a stimulant when pharmacological experiments have shown it to be inactive or even depressant. It has been suggested that probably camphor undergoes some metabolic changes in the body during absorption and a hypothetical stimulant substance is liberated. Efforts have been made by various workers to synthesize such products of camphor which could probably be formed inside the body between its absorption and excretion.

melting points, colour and structure of a new set of camphor compounds sent to us for pharmacological study :—

TABLE I.  
*Salts of camphor.*

Salts of camphor.	Colour and shape.	Melting point.	Optical rotation.	REMARKS.
(a) <i>Water-insoluble camphors.</i>				
d-camphor ..	White crystalline	178°C. to 179°C.	$[ \alpha ] \text{Hg}_{\text{gr.}}^{35^\circ} = +55.54^\circ$	Soluble in alcohol and glycerol. Action depressant throughout; chances of emboli; slight stimulation in diseased or artificially slowed heart.
l-camphor ..	Brown crystalline	178°C. to 179°C.	$[ \alpha ] \text{Hg}_{\text{gr.}}^{35^\circ} = -53.05^\circ$	
ll-camphor ..	White crystalline	..	$[ \alpha ] \text{Hg}_{\text{gr.}}^{35^\circ} = 0^\circ$	
borneol ..	White amorphous	204°C. to 205°C.	$[ \alpha ] \text{Hg}_{\text{gr.}}^{35^\circ} = -42.62^\circ$	
camphor quinone.	Deep yellow accicular.	199°C. to 200°C.	$[ \alpha ] \text{Hg}_{\text{gr.}}^{35^\circ} = -176.0^\circ$	do.
amphor ..	Deep yellow fine accicular crystals.	199°C. to 200°C.	$[ \alpha ] \text{Hg}_{\text{gr.}}^{35^\circ} = +175.7^\circ$	
amphor ..	Deep yellow amorphous creamy.	198°C. to 199°C.	$[ \alpha ] \text{Hg}_{\text{gr.}}^{35^\circ} = 0^\circ$	
(b) <i>Water-soluble camphors.</i>				
odio-iso-oso camphor	Deep yellow amorphous.	..	..	Loses its colour to light yellow.
dio-iso-oso camphor	Pale yellow	..	..	Inclined to be white.
o-iso-nitroso phor (race-	Pale yellow	..	..	Like above but slightly increased stimulation and rapid absorption.

There is no record in the literature regarding the difficulty of intravenous ministration of the insoluble isomers of camphor. Camphor is so sparingly soluble in water that intravenous injection of this drug in Locke's solution had to be given up as such large quantities had to be injected that the blood pressure rose to a considerable extent due to the amount of fluid injected and therefore obscured the real effects of the drug itself.

(a) *Insoluble camphors*.—Three solvents were used, i.e., alcohol (absolute), glycerol and olive oil. Control experiments with the pure solvents in all the three cases showed varying degree of pharmacological effect. Alcohol (50 per cent), even in doses of  $\frac{1}{2}$  c.c. administered intravenously to cats weighing 2 to 3 kilos, anæsthetized with chloralose, often produced cardiac embarrassment as shown by a sudden fall in the blood pressure and stoppage of its pulsation which gradually returned to normal after 3 to 5 minutes. The respiration was affected which also had an indirect effect on the circulatory system. The use of alcohol as a solvent had to be finally given up as the dilute forms of alcohol did not dissolve the camphor properly. Glycerol was next tried. It was found to be less toxic than alcohol but it was also not free from untoward effects. Half to one c.c. of glycerol administered intravenously under similar conditions as the above showed, sometimes later, a gradual fall in blood pressure which returned to normal gradually after 3 to 5 minutes. Olive oil,  $\frac{1}{2}$  c.c. to 1 c.c., administered under similar conditions in about 50 per cent of animals showed a slight and slow fall of blood pressure which gradually returned to normal. In the case of glycerol and olive oil, sometimes other effects like irregularity of heart beat and embarrassment of breathing occurred, due most probably to fine emboli formation in the pulmonary blood vessels and capillaries of the brain.

Finally, olive oil was preferred as solvent for the insoluble camphors for experimental work on animals. One per cent solution of the various camphor compounds in olive oil was prepared. The injection had to be given slowly with the normal saline running continuously from the burette into the vein, otherwise the oil floated back in the burette instead of going into the vein. The rubber-tubing connecting the burette to the femoral veins had also to be milked down after each injection so as to force out any oil that may be sticking in the lumen of the tube and is washed out again by letting more saline. Without these precautions the camphorated oil would stick in the tube or float back instead of completely entering the venous system. The effects produced by different camphor compounds are tabulated in Table II :—

TABLE II.

*Pharmacological action of camphor.*

Camphor.	Blood pressure.	Myocardio-graph.	Isolated heart.	Depressed heart.	Intestine volume.	REMARKS.
(a) <i>Insoluble camphors.</i>						
d	Fall ..	..	..	Slight stimulation.	Slight increase.	Heart depressed with quinine, emetine, hæmorrhage, etc.
dl	Fall irregular with big dose.	..	..	Nil	Big dose fall.	
I	Fall, less marked	Loss of tone	..	Nil	Slight increase.	
Borneol	Fall, not marked	Slight loss of tone.	..	Nil	do.	

TABLE II—concl'd.

Camphor.	Blood pressure.	Myocardiograph.	Isolated heart.	Depressed heart.	Intestine volume.	REMARKS.
(b) Insoluble camphors (camphor quinone).						
d	Slight stimulation, later on fall.	Depressed but irregular.	Temporary rise (marked).	..	..	(d) Frog's heart depressed, stimulation after a latent period.
dl	Slow and irregular fall.	..	do.	..	..	
l	Fall ..	..	do. (less).	..	..	
(c) Water-soluble camphors (sodio-iso-nitroso camphor).						
d	Less fall ..	Pulse 200-160	Tone increased 1-500,000 and 1-1,000,000	Temporary rise.	..	(d) Intrahepatically 1 per cent solution stimulation slowing, later on periodicity missing beats (frog's heart).
dl	Irregular ..	Pulse 200-180	Less.	Less.	..	
l	Pulsation imperceptible.	Pulse 200-180 irregular.	Coronary flow reduced, missing beat.	Less.	..	

In Sections (a) and (b) of the table it will be seen that the blood pressure is lowered in all cases with d-, dl-, and l-camphors. The quino-compounds showed a tendency of transitory stimulation which was followed by a fall which occurred gradually and was maintained. The frog's heart was depressed, but with d-camphor there was a slight improvement after a latent period. In the hearts of the cat and the rabbit when depressed artificially by giving doses of quinine, emetine, antimony or in cases of hæmorrhages and shock, etc., 2 mg. to 3 mg. of camphor per kilo body-weight produced a slight and temporary rise particularly in case of d-camphor. l-camphor produced the same effect but to a lesser degree; dl- and borneol camphor produced practically no remarkable effect. The rise of blood pressure was slight and temporary followed by a fall which did not reach such a low level as with ordinary camphors. The heart remained irregular.

(b) *Water-soluble camphors*.—Water-soluble camphor compounds in the form of sodium iso-nitroso camphors (d-, l- and dl- isomers) were used. These compounds were readily soluble in normal saline and yielded a clear pale yellow solution. The effect on the blood pressure, myocardiograph and the isolated heart was studied. A little more detailed action was studied on the hearts of frogs. The results are tabulated in Section (c) of Table II. It will be observed that the blood pressure is reduced though the effect is rapid and temporary in nature. The heart beats were

slowed but the muscular tone was increased in the isolated heart when it was perfused with dilutions of 1 in 500,000 to 1 in 1,000,000. The coronary circulation was reduced. The rabbit's heart was stimulated with 1 in 1,000,000 dilutions of d-camphor derivative but it was soon followed by depression; slowing and later irregularity of beats followed. The l-camphor derivative produced only a mild stimulation with 1 in 50,000 to 1 in 100,000. There was a fall of blood pressure in cat when 10 mg. of d-camphor derivative was injected intravenously. The blood pressure gradually returned to normal. This effect was practically absent in l- and dl-camphor derivatives. The pulse rate was reduced from 200 to 160 beats per minute with d-camphor derivatives. The other two isomers did not produce such a marked effect. The heart volume, the auricles and ventricular beats were not effected at first but bigger doses lead to dilatation and irregularity of rhythm. With d-camphor derivatives a periodicity was commenced. There was missing of beats both in the auricle and ventricle after every 16 beats. In the case of frog the action of these camphors was much more delayed. In some cases there was a slight stimulation augmenting the contraction and the number of beats. There appeared to be a slight stimulation when 0.1 c.c. of a 1 per cent solution of d-camphor derivatives was injected intrahepatically. The stimulation was more marked though much delayed in occurrence. After about 10 minutes a periodicity was noticed and there was a block in the conduction of impulses through the bundle. These effects disappeared after washing the heart with fresh saline. The other two isomers did not produce any marked effects.

#### DISCUSSION AND CONCLUSIONS.

The natural camphor and its synthetic isomers should be regarded, pharmacologically, as depressant to the isolated heart as well as in intact animals. When injected intravenously into cats and rabbits, camphor produces a gradual and prolonged fall of blood pressure. There may sometimes be noticed a mild stimulant effect after a prolonged perfusion. The isolated frog's heart (Straub's method) is depressed in various concentrations, while in weak concentrations and after a latent period the heart appeared to be slightly improved in its beats. The nutrient fluid, containing weak concentrations of camphor and which has been kept in contact with the heart or other living tissue for a certain length of time, if perfused through another isolated heart, improves it slightly. This shows that camphor probably when coming in contact with a living tissue or when it has remained in the body for a certain length of time undergoes some biochemical changes and then produces diametrically opposite effects on the heart. Similar changes may possibly be taking place inside the body after the administration of camphor and may possibly explain its marked cardiac stimulant effect when used clinically. During the pharmacological experiments the metabolic and oxidation processes may not take place so rapidly with insoluble camphors and therefore the stimulant effects are not manifested. Intra-arterial injections are known to be more depressant than intravenous injections. The hypothetical products, possibly an intermediate between camphor and its excretion product in combination with glucuronic acid, were synthesized and studied by Tamura, Kihara and Ishidate (1930 to 1934). The stages of oxidation of camphor inside the body possibly are: (1) The camphoral (p-o-oxy-camphor) which was found to be slightly stimulant after an initial depression and latency. (2) The next product in order of formation is a glucoside of p- and o-oxy-camphor which

experimentally produced no stimulant effect on the heart. The other possible intermediate products of metabolism are o-oxo-camphor (o-Diketo camphene) and p-oxo-camphor (p-Diketo camphene). Of these two, while the former has no effect the latter is markedly stimulant to the perfused frog's heart. Intravenous injection of this latter product in frogs and rabbits, in doses of 0.1 c.c. of 1 per cent solution, produced immediate and marked stimulant effects. The action of camphor inside the system may thus be : (1) The original camphor and its final oxidation products are directly depressant to the isolated heart and the circulatory system. (2) The intermediate products of oxidation particularly p-oxo-camphor and o-oxo-camphor produce a marked stimulation on the heart. (3) The immediate stimulation of camphor after an injection may be merely a local reflex effect. (4) The secondary maintained and prolonged effect may be due to the slow absorption and gradual conversion of camphor into intermediate oxidation products which supply stimulation to the exhausted heart. This chemical synthesis inside the body occurs most probably inside the liver. It has been observed that if the liver is damaged or is not functioning properly, camphor does not produce its stimulant effects, but on the other hand has been reported to produce toxic effect in the individual (Sabatine, 1925). Intravenous and intrahepatic injections of camphor into frogs produced a slight stimulant effect on the heart, whilst the intra-arterial injection was always depressant in action.

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## SOME INORGANIC PREPARATIONS OF THE INDIAN INDIGENOUS MEDICINE.

### Part II.

#### BANGA BHASMA (CALCINED TIN).

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TIN occurs in nature in combination with oxygen in the mineral Cassiterite or Tinstone, which is more or less a pure form of tin dioxide,  $\text{SnO}_2$ . It is found also as tin pyrites and sometimes as a silicate, but the principal source of tin is the dioxide. Tin has been known in India from ancient times. Its vernacular name is *Banga* in Sanskrit and *Rāṅg* in Hindi and Bengali. It is said to occur in the Peninsular India and in the district of Hazaribagh in Bihar. The chief source of tin in and near India is Burma, Tenasserim and Malaya Archipelago.

Like other metals which have been used in old Hindu medicine, tin has been used in the form of a crude oxide which is prepared by a complicated process of so-called 'purification', but which really reduces it to a state of impure oxide.

The following process is generally adopted for this purpose: Metallic tin is heated in an iron pan until it is melted and the molten mass is poured into the milky juice of Arka (*Calotropis gigantea*). It is then re-melted with one-fourth of its weight of Yabaksara (impure carbonate of potash) and powdered husk of tamarind is added to it. The whole mass is stirred well with an iron-rod till it is reduced to a very fine powder. The powder is then washed with cold water and dried over a gentle fire. Another method of preparation is to heat the metal on fire in an iron pot; when molten, powdered turmeric, Jirak (*Cuminum cyminum*), Trifala, i.e., the three fruits Haritaki (*Terminalia chebula*), Bahera (*Terminalia bellerica*) and Amlaki (*Phyllanthus embelica*), powder of Aswath (*Ficus religiosa*) and tamarind barks are put in one after the other and stirred. The next powder is only put in when the one previously added is thoroughly burnt. The product thus obtained is a greyish white fine powder and is known as *Banga Bhasma* or ash of tin.

According to well-known Ayurvedic physicians, *Banga Bhasma* is used in the following diseases with different vehicles (Anupana), usually in combination with other mineral preparations like the Bhasmas (ashes) of gold, silver, zinc, iron, etc. Sometimes it is used alone. Its main indication is in inflammatory and suppurative conditions of stomach, urethra and other mucus surfaces. It is believed to be a general tonic and alterative and is often combined with *Silajatu* and *Abhra Bhasma* for this purpose. Its chief uses are in diabetes, spermatorrhœa, gonorrhœa, anæmia, asthma, gastric ulcer and in various skin diseases. The dose is from one to four grains.

We analysed a sample of *Banga Bhasma* supplied by the Kalpataru Ayurvedic Pharmacy of Calcutta. The sample was a dull-grey amorphous powder with a slightly metallic and saline taste. It was soluble in hot water to the extent of 1·12 per cent. The chemical composition found as the result of qualitative and quantitative analyses is given below :—

	Per cent.
Oxide of tin, $\text{SnO}_2$ .. ..	82·94
Silica, $\text{SiO}_2$ .. ..	6·38
Iron and alumina, $\text{Fe}_2\text{O}_3$ , $\text{Al}_2\text{O}_3$ .. ..	2·96
Lime, $\text{CaO}$ .. ..	1·92
Magnesia, $\text{MgO}$ .. ..	0·69
Potash, $\text{K}_2\text{O}$ .. ..	2·96
Soda, $\text{Na}_2\text{O}$ .. ..	0·45
Chlorides .. ..	0·11
Moisture .. ..	0·89
Other constituents .. ..	0·70
TOTAL ..	100·00

The solubility of *Banga Bhasma* in dilute hydrochloric acid of a strength approximating that found in gastric juice was also studied. For this purpose 2·0 g. of the *Bhasma* were digested in 200 c.c. of 0·3 per cent of hydrochloric acid at a temperature of 37°C. for 24 hours. The total solubility was 7·726 per cent. The soluble portion when analysed quantitatively was found to have the following composition :—

	Parts.
Oxide of tin, $\text{SnO}_2$ .. ..	1·060
Silica, $\text{SiO}_2$ .. ..	0·342
Lime, $\text{CaO}$ .. ..	2·072
Iron oxide, $\text{Fe}_2\text{O}_3$ .. ..	0·243
Aluminium oxide, $\text{Al}_2\text{O}_3$ .. ..	0·137
Magnesia, $\text{MgO}$ .. ..	0·371
Potash, $\text{K}_2\text{O}$ .. ..	2·967
Soda, $\text{Na}_2\text{O}$ .. ..	0·424
Chlorides .. ..	0·110
TOTAL ..	7·726

#### TIN IN PHARMACOLOGY AND THERAPEUTICS.

Experiments have shown that when soluble salts of tin are given to animals, a small quantity is absorbed and accumulates in the tissues and tin appears in the urine. When soluble salts are given by subcutaneous injections, elimination is slow

and occurs mainly by the alimentary tract, but somewhat by the urine also. Diuresis results after administration of small doses but large doses have a deleterious effect on the kidneys and produce pathological changes in this organ. Large quantities of tin are retained in the body after administration of which 20 to 25 per cent is in the skin and 5 per cent in the liver.

It will be seen from the data given above that appreciable quantities of oxide of tin occurring in the *Bhasma* will be dissolved in the physiological acid of the gastric juice and will be absorbed into the system. Like other heavy metals small quantities of tin have a stimulant action on the central nervous system and also on hæmopoietic system and in this way have a general stimulant action and may be beneficial in such conditions as diabetes. The diuretic action is beneficial in chronic gonorrhœa and possibly traces of the metal in the urine may have some inhibitory effect on the organism responsible for this disease.

The action of tin on the heart is like that of arsenic and is probably through the vagus. It is possible that in its beneficial effects in asthma it may act by depressing the vagi in the same way as arsenical compounds like soamin do.

The use of tin in the treatment of skin diseases by the Ayurvedic physicians is rather interesting in view of the fact that there is a tendency for the metal to accumulate in the skin. It has been observed that workers in tin mines do not suffer from furuncles and based on this observation *stannoxyol* was introduced which has been successfully tried in patients suffering from furunculosis.

It may be worth while extending these trials in the treatment of such chronic and persisting diseases as eczemas, psoriasis, etc.

The nervous system is especially sensitive to this metal and large doses may produce peripheral neuritis, excitability and sclerosis of the brain or the spinal cord.

The therapeutic effects of tin compounds, however, are not so powerful as some of the compounds of other metals in use in Western medicine. Further investigations were therefore not considered necessary.

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## THE HUMORAL TRANSMISSION OF THE EFFECTS OF CARDIAC VAGUS AND SYMPATHETIC STIMULATION BY DRUGS.

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LOEWI (1921) showed that stimulation of the vagus produced some chemical substance which if allowed to pass on into a fresh heart produced in the second heart the same effect as if the vagus nerve of the latter had been stimulated. He also stimulated the sympathetic nerve to an isolated perfused frog's heart and could demonstrate some other chemical substance in the lumen of the ventricle, which when transferred to another heart produced in the second the effects of sympathetic stimulation. These findings were corroborated later on by other workers including Finkleman (1930) who recorded the spontaneous movements of two separate suspended strips of isolated small intestine, through the lumen of which saline was perfused from one to the other, and demonstrated thereby that stimulation of the sympathetic fibres to the first caused the relaxation of both the pieces (the second after a short latent period) and thus proved that some chemical substance formed in the first by the sympathetic stimulation was responsible for the same effect in the second. Similarly, Cannon *et al.* (1931) found that stimulation of the sympathetic nerves to the skin of cat's tail caused the liberation of a chemical substance with adrenaline-like effects, into the blood-stream. That a similar substance was liberated during life when the sympathetic nerves were stimulated, was also clearly demonstrated by them. Bain (1932) described a method of demonstrating humoral transmission of the effects of cardiac vagus stimulation in the frog and showed that when the vagus of the donor heart was stimulated for forty seconds the heart stopped in about fifteen seconds with slight slowing of the recipient heart which also came to a standstill after another fifteen minutes or so. He confirmed his finding regarding the liberation of the vagus substance on stimulation of the vagus nerve to the donor heart by allowing perfusion fluid to flow from it to an isolated stomach whose movements were also simultaneously recorded. The stomach gave a contraction of relatively large amplitude, attaining its maximum rapidly when the vagus nerve to the heart was stimulated for about 15 seconds causing stoppage of the heart; on discontinuing the vagus stimulation the stomach relaxed again.

From all these experiments there seems to be little doubt regarding the production of vagus or accelerans substance in the frog's heart caused by the stimulation of vagus and sympathetic respectively by electrical stimulation. This paper attempts to show whether there is production of any chemical substance in the heart by stimulation or inhibition of the vagus or the sympathetic by means other than electrical stimulation, e.g., certain drugs that act on these nerves.

#### EXPERIMENTAL.

Frogs were used for the experiment and heart to heart perfusion was done by Bain's (*loc. cit.*) method, slightly modified (*see* Diagram). The inflow cannula (*E*) to

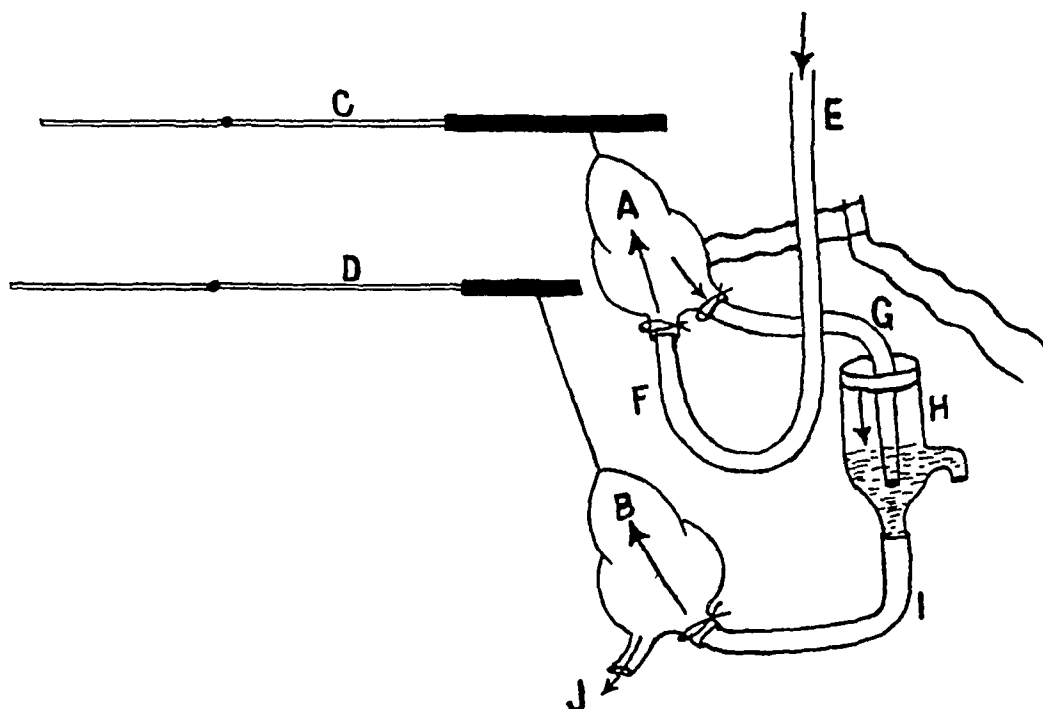


DIAGRAM OF THE APPARATUS.

- A — Donor heart.
- B — Recipient heart.
- C and D — Levers to which A and B are attached.
- E — Inflow tube to the donor heart.
- F — Cannula into inferior vena cava.
- G — Outflow tube from the donor heart.
- H — Glass cannulated tube.
- I — Inflow tube into the recipient heart.
- J — Outlet from the recipient heart through a cut into the aortic arch.

the donor heart was introduced through the inferior vena cava instead of through the right auricle. The outflow tube (*G*) from the donor heart was introduced into the first part of the arch of the aorta (with its branches ligatured) and passed into the glass cannulated tube (*H*). From the nozzle of the latter a venous cannula

connected by a very small piece of rubber-tubing (I) went into the inferior vena cava of the recipient heart. A cut in the aortic arch formed the outlet for the fluid from the second heart. The fluid used for perfusion was modified Ringer's solution for frogs (Macleod, 1930). The drugs used for the experiment were adrenaline chloride (P. D.), ergotoxin (B. W.), acetylcholine (B. D. H.), insulin (Lilly brand), pilocarpine nitrate, nicotine and atropine sulphate. The drugs were all dropped on the donor heart so as to stimulate or paralyse the sympathetic, the vagus or the ganglion as the case may be.

## RESULTS.

(See Cardiographs.)

*Adrenaline.*—When 3 drops of adrenaline chloride (1 in 1,000) were dropped on the donor heart the rate as well as the amplitude of contraction were increased. The recipient heart which was working rather slowly with some irregularity, not only became regular but also showed much improvement in rate as well as the force of contraction (Fig. 1).

*Ergotoxin.*—Ten drops of ergotoxin (0.065 per cent solution) caused some slowing of the heart. The recipient heart also showed same effect with diminution in amplitude. Curiously enough, ergotoxin had not much appreciable effect on a fresh heart and the effect manifested itself when the heart was working for some time (Fig. 2).

*Nicotine.*—Five drops of 1 per cent solution caused slowing with much diminution in amplitude of contraction of the donor heart, which was later on followed by much improved beats (in amplitude as well as in rate). The same effect was also noticed on the recipient heart (Fig. 3).

*Acetylcholine.*—Fifteen minims of 5 per cent solution when dropped on the donor heart caused complete stoppage of the heart which could be revived only by a few drops of atropine (10 drops of 0.5 per cent solution) by counteracting the action of the former on the heart. The recipient heart too showed analogous effects (extreme slowing with lowering of the amplitude), which became improved again when the donor heart was acted upon by atropine (Fig. 4).

*Pilocarpine.*—Ten drops of 1 per cent solution after a short latent period produced slight slowing of the donor heart. The same was also noticed on the recipient heart with occasional missing of the beats (Fig. 5).

*Insulin.*—Five units when dropped on the heart caused slowing in the rate of contraction with some irregularity in the donor heart. The recipient heart was also slightly slowed down with diminution in amplitude, which regained its former height as the donor heart was partly shaking off the effects of insulin (Fig. 6).

*Atropine.*—0.5 per cent solution had very little effect when applied to a fresh heart, nor was the recipient heart affected in any way. But it had its effects markedly on a heart already slowed down under the influence of some other antagonistic drug, e.g., acetylcholine and the recipient heart was also affected similarly (Fig. 4). In the same way it can also counteract, when previously administered, the effects of the antagonistic drugs on the donor and recipient hearts, to some extent.



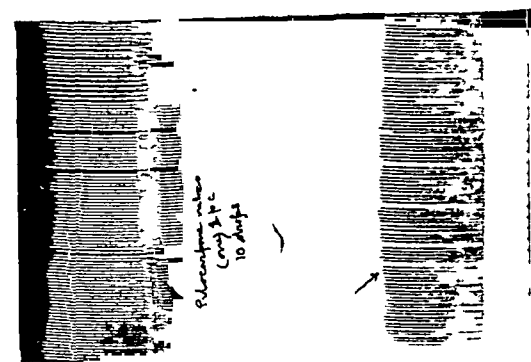
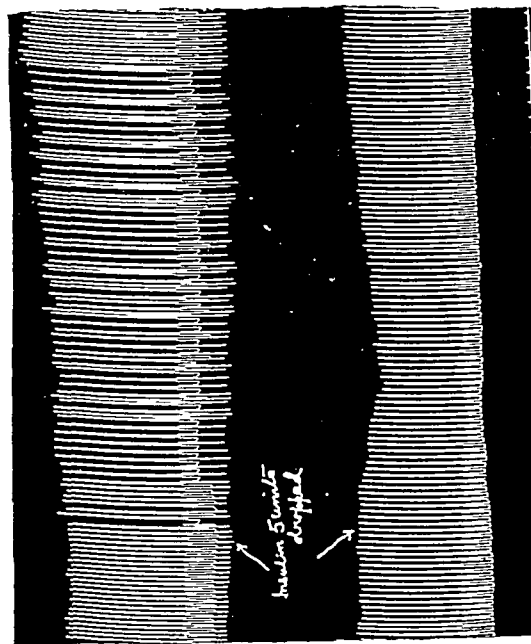
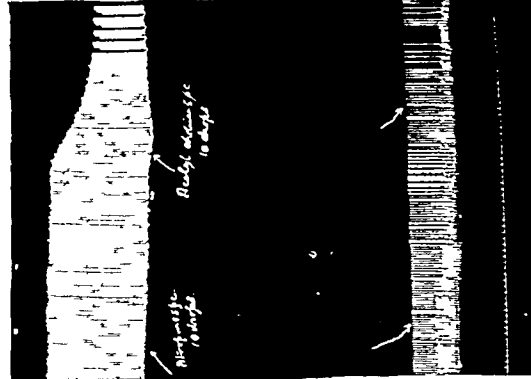
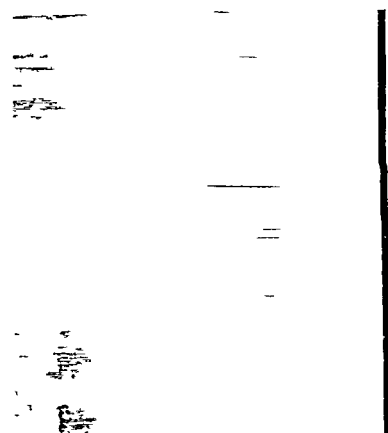
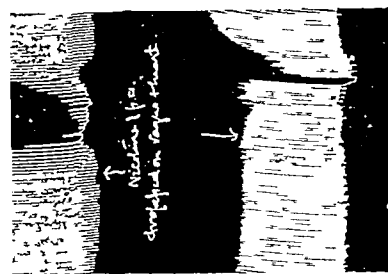
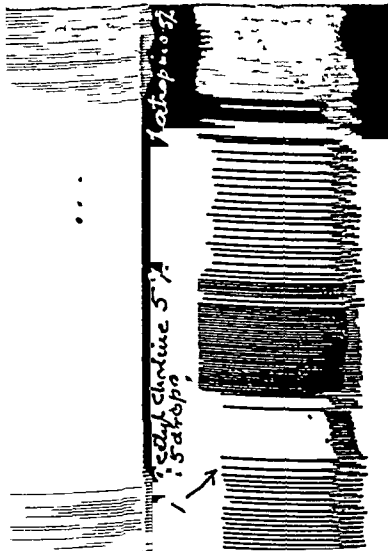


Fig. 7.

Fig. 6.

Fig. 5.

Fig. 1—Upper, donor heart; lower, the recipient heart, time 5 seconds. Adrenaline (1 in 1,000), 3 drops on the donor heart—improvement in rate and amplitude with disappearance of the irregularity in the recipient heart. Fig. 2—Ergotoxin, 10 drops of 0.065 per cent on the donor heart—slowing in rate and diminution in amplitude of the recipient heart corresponding to the effects on the donor heart. When the donor heart was partially recovering from the effects of the drug the recipient heart too showed improvement in amplitude. Fig. 3—Five drops of 1 per cent solution of nicotine—extreme slowing with diminution in amplitude of the donor heart followed by improvement. Similar effect on the recipient heart also followed 1% drops of 1% per cent solution—complete stoppage of the donor heart followed by 10 drops of 0.2 per cent atropine—recipient heart also stopped, but it then recovered and amplitude increased. Fig. 4—1% drops of 1% per cent atropine, slowing of 1 per cent on the donor heart and 1 per cent on the recipient heart. Fig. 5—1% drops of 1% per cent atropine, slowing of 1 per cent on the donor heart and 1 per cent on the recipient heart. Fig. 6—1% drops of 1% per cent atropine, slowing of 1 per cent on the donor heart and 1 per cent on the recipient heart. Fig. 7—1% drops of 1% per cent atropine, slowing of 1 per cent on the donor heart and 1 per cent on the recipient heart.

Fig. 7 shows only some diminution in amplitude with slowing of the hearts after 10 drops of acetylcholine, on a heart previously acted upon by atropine (10 drops) even without any appreciable effect, the recipient heart too showing slowing in rate.

#### DISCUSSION.

From the above results it is evident that the drugs that act on the autonomic nervous system (sympathetic, para-sympathetic and ganglia) are capable of producing some chemical substance in a heart which if allowed to pass on into another heart will affect the latter similarly. Drugs that act on the sympathetic by stimulation, as adrenaline, produce something in the heart that causes acceleration and augmentation as well in the recipient heart. The reverse effects are produced by the inhibitor of the sympathetic, e.g., ergotoxin. Nicotine first stimulates and then paralyses the ganglia; this also produces something in the donor heart that has at first a slowing and then stimulating action on the recipient heart. Acetylcholine, pilocarpine, insulin (Pal and Prasad, 1934), etc., all stimulate the vagus endings and consequently produce the vagus substance which leads to effects on the second heart similar to those after stimulation of the vagus by electric current. Atropine, being the paralysing agent of the vagus terminals, has the reverse effect especially after the action of the antagonistic drugs and to minimize the action of such drugs when administered previously.

Curiously enough, the drugs that have a stimulating influence on the sympathetic or the vagus have got more marked effects than their physiological antagonists. In this respect ergotoxin and atropine, though less effective, still produce positive results under certain conditions. Some observers such as Stefan (1928), have not been able to find any positive result with atropine and ergotamine. The reason probably is that the effects of negative stimulation of the nerves do not come in as spontaneously and quickly as those of positive stimulation. So it can be said definitely that as vagus substance and 'sympathin' (Cannon *et al.*, *loc. cit.*) can be produced by stimulation of the vagus and sympathetic respectively, the drugs that stimulate the nerves can also produce allied substances which when transferred to another heart will lead to corresponding effects of vagus and sympathetic stimulation respectively. Even the antagonistic drugs such as ergotoxin and atropine that paralyse the sympathetic and vagus respectively, if allowed to act on a donor heart are capable of producing anti-vagus and anti-accelerans substances (anti-sympathin?) with typical effects of paralysis of the respective nerve terminals on the recipient heart.

As the method of heart to heart perfusion described in the text ensures a constant flow under a constant pressure, the effects on the recipient heart can be taken without any doubt as due to some chemical substances produced in the donor heart under the influence of various drugs.

#### CONCLUSION.

Drugs acting on the vagus and sympathetic by stimulation are capable of producing vagus and accelerans substances respectively in the heart. The antagonistic drugs that have a paralysing action on the nerve terminals can also produce anti-substances when they are allowed to act on a heart. The negative stimulation

produced by them is undoubtedly less effective for the liberation of specific chemical substances than the positive stimulation. Drugs acting on the ganglia also produce some corresponding chemical substance in the heart.

#### SUMMARY.

Humoral transmission of the effects of drugs acting on the autonomic nervous system has been definitely proved.

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## A STUDY ON THE ACTIVATION OF TISSUE-GROWTH (IN VITRO) WITH COBRA VENOM.

BY

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### INTRODUCTION.

THE cobra venom is said to have remarkable therapeutic properties in many pathological conditions, and in small doses it is believed to have marked stimulating effects on certain tissues. In large doses it is also known to affect certain tissues and its powerful effect on the nervous tissue in particular is well known. The peculiarity of the action of this venom is that in very minute doses it has stimulant effects on the nerve cells and is useful in therapeutics, but in larger doses the action is positively harmful, causing paralysis and death of these cells. Although all these facts have been known, the way in which this action is brought about and the nature of such action have still remained obscure. The present work was undertaken to study the effect of this venom on the growth of tissues cultivated *in vitro* and thereby to throw some light, if possible, on its mode of action.

### EXPERIMENTAL PROCEDURE.

The tissues were obtained from chick's embryo ten days old. Freshly laid eggs were carefully incubated at 40°C. for ten days, after which the embryo was removed with aseptic precautions. The tissue from the choroid coat of the eye was always used in these experiments since this tissue has been found to grow well under artificial conditions, and being of a membranous nature the growth can be easily pursued. Very small pieces of this tissue (of the size  $\frac{1}{2}$  mm. sq.) were taken with edges clearly cut. The clear-cut edges are absolutely essential in these experiments since with rough edges it is difficult to follow the progress of the growth.

Before actually proceeding with the experiments, embryo extract was prepared by cutting the remainder of embryo in small fragments and by stirring these with a

ramrod in Pannet's solution. The clear supernatant liquid obtained after centrifugalization gave the necessary extract. The chick plasma was also obtained from blood drawn from the wing vein of a cock. As a precaution against clotting, the whole operation of drawing blood and separating the plasma was carried out with a cold paraffined syringe and cold test-tube. The plasma was also stored in the cold.

After these preliminary operations, hanging drop preparations on coverslips with a drop of plasma and the tissue extract were made in which the choroidal tissue was explanted. These were incubated at 37°C. and observations were made at intervals of 24 hours up to 60 hours and the results noted.

In experiments with cobra venom explants were made as before, the only difference being that a drop of the venom solution of a given concentration prepared in Pannet's solution was mixed with it in addition. Controls in this case were kept by adding a drop of Pannet's solution in the explants instead of a solution of venom.

### RESULTS AND DISCUSSION.

The results obtained have been given in the Table. A perusal of this table will show that with cobra venom in dilution from 1 in 1,000 to 1 in 20,000, the growth was definitely inhibited, while in dilutions of 1 in 50,000 and upwards the growth appeared to be stimulated. In all the series of more than 150 experiments for each dilution uniform results were obtained.

TABLE.

*Showing the relative growth of tissues.*

#### COBRA VENOM DILUTIONS.

Experiment number.	1 in 100.			1 in 1,000.			1 in 10,000.			1 in 20,000.			1 in 50,000.		
	Number of control.	Number of experiment.	Results.	Number of control.	Number of experiment.	Results.	Number of control.	Number of experiment.	Results.	Number of control.	Number of experiment.	Results.	Number of control.	Number of experiment.	Results.
1	5	5	—	5	6	—	7	9	—	6	5	±	6	5	+
2	10	12	—	10	8	—	6	7	±	6	9	±	8	12	±
3	8	10	—	12	11	—	3	4	—	10	6	±	8	10	+
4	9	12	—	9	12	*	9	6	—	7	8	—	9	12	+
5	10	16	—	10	8	—	8	5	±	9	12	±	10	6	±
6	10	12	*	15	12	—	12	8	—	7	3	—	8	8	+
7	12	15	—	6	8	—	6	7	—	5	8	—	10	8	+
8	9	6	—	9	12	—	5	5	±	9	12	±	12	12	+
9	8	10	—	5	7	±	9	8	*	7	10	+	14	10	+
10	5	7	..	4	6	—	7	4	—	6	5	±	9	8	+

— less growth in comparison to control.

± doubtful increase of growth.

+ growth increased compared with the control.

\* These experiments were vitiated due to contamination and are therefore inconclusive.

It will be observed from the Graph that in dilutions of 1 in 100 or in 1 in 1,000 the growth is totally inhibited, while in dilutions of 1 in 10,000 and 1 in 20,000 there is slight growth although it is much less than the control. But in dilutions of 1 in 50,000 or in 1 in 80,000 the growth is much greater than that of the control. The curve for the growth for the dilution of 1 in 80,000, however, runs below that for 1 in 50,000 at which dilution, therefore, the growth seems to be at its maximum. The Curve showing growth with dilution is given.

It is well known that for the growth of a tissue two factors are mainly responsible; the first being the support on which the tissue grows and the second being the presence of certain substances in the medium which promote growth. Carrel (1912) found that the best support for growth is the fibrin network of the blood plasma. Carrel and Baker (1926) explained the stimulating action of the embryo extract as due to the proteolytic products formed by the hydrolysis of proteins by the cell enzymes. He also showed that the higher proteolytic products such as proteoses, etc., have a stimulating action upon the tissue-growth, whereas peptone and other smaller split products, although appear to furnish some nutrient material, do not, however, cause rapid proliferation characteristic of proteoses; on the other hand, they often prove toxic for tissue cells, especially when present in larger amounts. These authors also studied the action of the hydrolytic products of commercial fibrin and produced further evidence in support of their views.

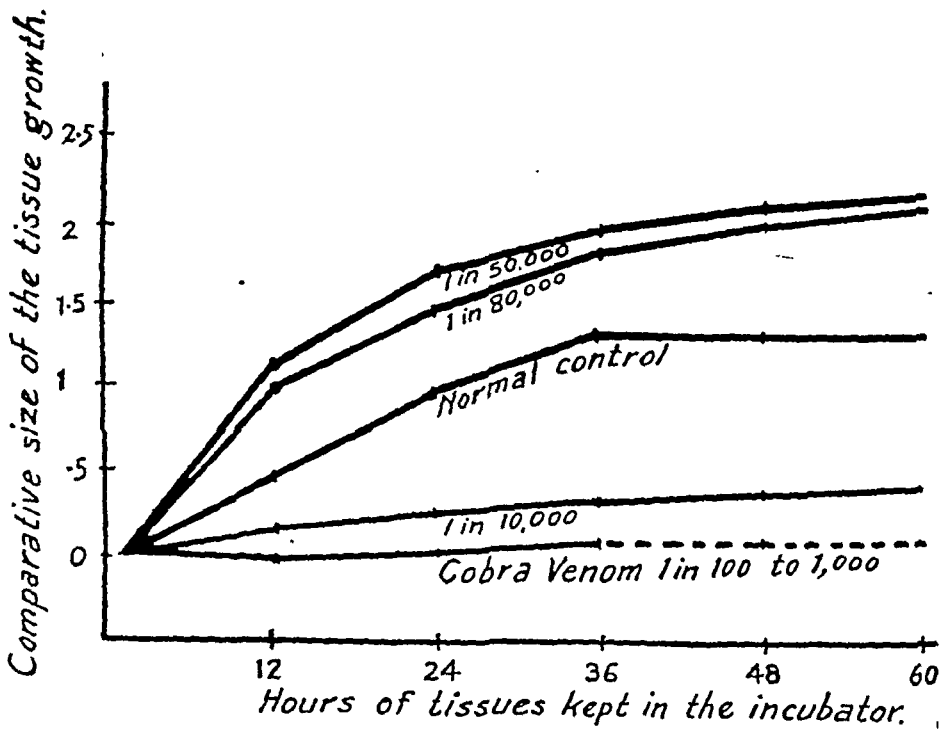
Cobra venom, since it affects the growth of tissues, must influence one or other of the two factors responsible for tissue-growth. It may have influence either on the fibrin network which acts as a support or it may act by changing the medium only.

As early as 1902 Flexner and Noguchi observed that the cobra venom could digest fibrin which forms the support for the growth of tissues in the present case. Since then different authors established the fibrinolytic properties of venom in general and cobra venom in particular. The recent work of Chopra and Roy (to be shortly published) has definitely established the same point and has given a comparative study of the more common snake venoms so far as their proteolytic actions are concerned. Martin (1905) also found fibrin ferments in various venoms and he was of opinion that these ferments were probably responsible for thrombosis.

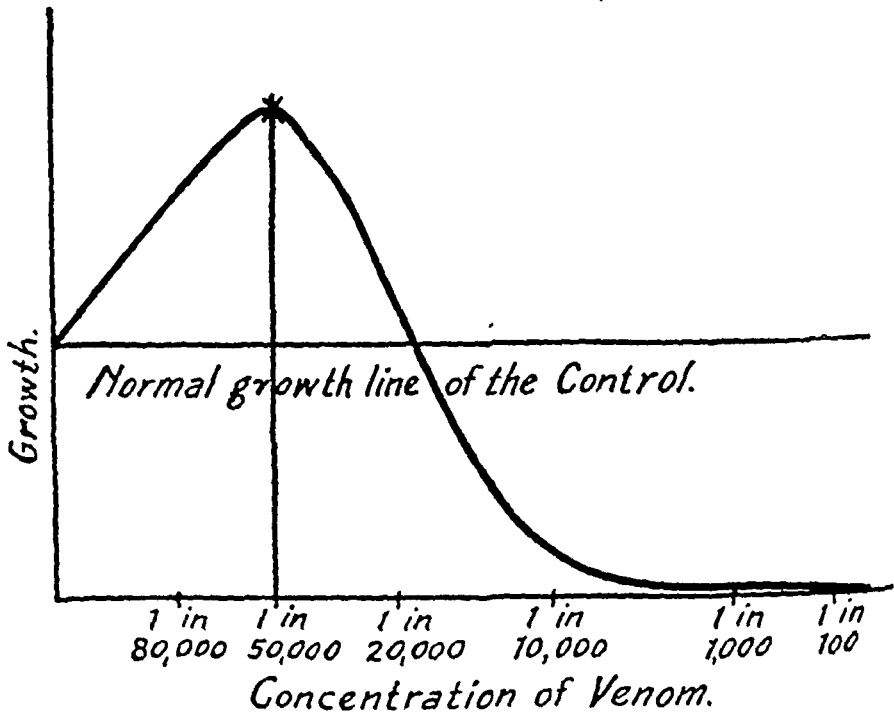
In the light of these contributions it is not difficult to form an idea as to how venom affects the growth of tissues. In the first place it stimulates the formation of thrombin (Houssay and Sordelli, 1920) and thereby the production of fibrin. Secondly, the enzymes present in the venom also have a hydrolytic action on the fibrin. Thus the two antagonistic actions proceed side by side. In course of some experiments carried out in this laboratory on the coagulation of blood it was observed that the cobra venom promoted coagulation of blood in small concentrations only, whereas in larger concentrations the blood did not coagulate at all. This evidently shows that in smaller concentrations the venom promotes the formation of thrombin more than the destruction of fibrin, while in larger concentrations the fibrinolytic action is more pronounced and for this reason the blood cannot coagulate.

The importance of fibrin in the present experiments on the cultivation of tissues consists in this that it forms the support on which the tissue grows. At low concentrations wherein the formation of fibrin is believed to be promoted as evidenced by increased coagulation of blood, the growth of the fibroblasts is evidently at its

GRAPH.



CURVE.



maximum. On the other hand at concentrations where coagulation experiments show a maximum fibrinolytic action of the venom the growth of tissues is inhibited. This parallelism of the tissue-growth with the formation and destruction of fibrin seems to be very suggestive, inasmuch as the fibrin network appears to be a strong influencing factor for the growth of tissues, in presence of cobra venom.

Over and above this there is another factor which seems to be equally important. From the work of Carrel and Baker (*loc. cit.*) it is evident that higher proteolytic products stimulate the growth of tissues, while lower products may prove toxic and consequently inhibit growth. It is not, however, unlikely that higher split products like proteoses are formed only when the concentration of venom is small, whereas with larger concentrations of venom other subsequent products are possible. In the light of this in lower concentrations a stimulation of growth might be expected due to presence of proteoses, whereas in higher concentrations an inhibition is possible. The fibrin would thus appear to be of great importance for the growth of tissues. The support which is formed of fibrin network has therefore a strong influence on the cultivation of tissues.

Last but not least there are also several physico-chemical alterations that are brought about by the mere introduction of the venom or by its proteolytic reactions. These physico-chemical factors no doubt act as auxiliary factors in course of the growth of tissues, but the direction in which they act is not yet very clear.

#### SUMMARY AND CONCLUSIONS.

1. Cobra venom in higher dilutions stimulates the growth of tissues *in vitro*, while at lower dilutions it inhibits growth.

2. This promotion and inhibition of growth have been explained as due to the different types of enzyme action on fibrin. At higher dilutions the formation of fibrin is promoted by fibrin ferments for which it is likely that the growth is also promoted. At lower dilutions the fibrinolytic action of the venom is more pronounced for which the support on which the tissue grows is likely to be affected, and in consequence the growth is inhibited.

3. The proteolytic products of fibrins are also responsible for growth. At higher dilutions the growth is promoted due to the formation of proteoses, while at lower dilutions smaller split products probably prove toxic to the cells.

4. The physico-chemical changes brought about by the introduction of venom and by its proteolytic activity must also have an effect, but the direction in which such changes act are not known.

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## THE ACTION OF RATTLESNAKE AND MOCASSIN VENOMS AS COMPARED WITH INDIAN VIPER VENOMS.

BY

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THE receipt of a small supply of the venoms of the American vipers *Crotalus adamanteus* (rattlesnake) and *Ancistrodon piscivorus* (mocassin) has enabled a comparison to be made between the action of these venoms and that of the Indian vipers *V. russellii* (daboia) and *Echis carinata* (phoorsa), the results of the study of which have already been reported (Taylor *et al.*, 1935a, b, and c).

### *CROTALUS ADAMANTEUS* VENOM.

#### *Neurotoxic action.*

The rapidly fatal action of the intravenous injection of the venom in standard pigeons of 300 g. weight was taken as an indication of the neurotoxic content of the venom. A series of pigeons were given intravenous injections of a solution of the dried venom in a strength of 1 mg. per c.c.

Pigeon No. 1. Dose 0.1 mg. Symptoms of shock, vomiting after 27 minutes, recovery.

„ No. 2. Dose 0.15 mg. Similar symptoms, recovered.

„ No. 3. Dose 2 mg. Immediate symptoms of shock, pigeon collapsed and lying on side, slow respiration, slight convulsions, died in 16 minutes.

„ No. 4. Dose 0.25 mg. Similar symptoms, died in 17 minutes.

The minimum lethal dose can be taken to be about 0.2 mg. which is 20 times that for *daboia* venom (0.01 mg.) and four times that for *echis* venom (0.05 mg.)

At post-mortem examination the two pigeons that died showed intense congestion of the vessels of the splanchnic area and engorgement of the abdominal viscera. The left side of the heart was empty and the right side distended. No clots were noted in the vessels and the blood did not coagulate on standing overnight in paraffined tubes. The cause of death would appear to have been an extreme fall in blood pressure following paralysis of the splanchnic capillaries, the action being the same as that described by Chopra and Chowhan (1934) in the case of *daboia* venom poisoning.

#### *Neutralization of neurotoxin by daboia antivenene.*

In previous work with viper venoms we have found that all those so far examined, including some Indian, European and African vipers, possess specific neurotoxic properties and that their *neurotoxins* were not neutralized by the heterologous antivenenes which were available for testing. Neutralization of the immediate lethal effect of the rattlesnake venom by the antivenene prepared at Kasauli, of which 1 c.c. unconcentrated serum neutralizes 1 mg. of *daboia* venom and 1 c.c. concentrated neutralizes 4 mg., was tested.

The following mixtures were injected intravenously in standard pigeons:—

0.4 mg. rattlesnake venom (= 2 m.l.d.) + 1 c.c. unconcentrated antivenene—died in 12 minutes.

0.4 mg. rattlesnake venom (= 2 m.l.d.) + 1 c.c. concentrated antivenene—died in 17 minutes.

In a proportion equivalent to ten times its titre against *daboia* venom the serum had no neutralizing action on rattlesnake venom.

An unconcentrated *echis* antivenene was also available for testing but this was only of low titre, 1 c.c. neutralizing 0.5 mg. of the *echis* venom. An intravenous injection of a mixture of 2 c.c. of the antivenene and 0.4 mg. (= 2 m.l.d.) of rattlesnake venom caused the death of 2 standard pigeons in 5 minutes. This antivenene did not neutralize the neurotoxic action of the venom in proportion of two and a half times its titre. These results are in line with our previous experience of the specificity of this action of viper venoms.

#### *Hæmorrhagin content.*

The *hæmorrhagin* content of the venom was determined by intracutaneous injection on the skin of the abdomen of white rabbits. 0.1 c.c. of a series of dilutions of the venom was injected and the production of hæmorrhage noted at the end of one hour.

Venom dilutions	..	1/10,000	1/50,000	1/100,000	1/500,000
Results	..	+	—	—	—

The minimum skin hæmorrhagic dose was 0.01 mg. which is the same as has been found for the venoms of *Echis carinata* and *V. berus* and one-tenth of that found for *daboia* venom. *Crotalus adamanteus* venom thus comes into the group of venoms of high *hæmorrhagin* and low *neurotoxin* content.

*Neutralization of the hæmorrhagin by daboia antivenene.*

The hæmorrhagins of all viper venoms which we have previously examined were found to be neutralized by antivenenes prepared against different viper venoms (Taylor and Mallick, 1935a). Neutralization of hæmorrhagin of rattlesnake venom with our antivenene was tested by intradermal injection of mixtures of the venom and the serum. The following doses were given :—

Antivenene + Venom solution.	Dose.	Number of skin doses.	Result.
1 c.c. + 1 c.c. (= 0.1 mg.)	0.2 c.c.	1	—
1 c.c. + 1 c.c. (= 0.2 mg.)	0.2 c.c.	2	+
.. 1 c.c. (= 0.1 mg.)	0.1 c.c.	1	+

The hæmorrhagin of the venom was neutralized in accordance with the titre of the serum and in relation to the relative hæmorrhagin content of the *daboia* and rattlesnake venoms.

The neutralization of the hæmorrhagic action of the venom of *daboia* antivenene was further tested *in vivo*. It had been observed during experiments with *echis* venom (Taylor and Mallick, 1935b) which in *neurotoxin* and *hæmorrhagin* content is very similar to rattlesnake venom, that the results of doses which are not rapidly fatal were mainly of hæmorrhagic nature and could be largely prevented by the use of heterologous viper antivenene. This was tested in rabbits, one rabbit being given 3.0 mg. of the rattlesnake venom subcutaneously on the thigh and another the same dose along with 7.5 c.c. of concentrated antivenene, which is the calculated dose for neutralization of its hæmorrhagin. After 24 hours the control rabbit showed extensive hæmorrhagic swelling over the whole thigh spreading into the scrotum and on to the lower part of the abdomen. The skin of the scrotum sloughed on the following day and the animal was chloroformed. On examination a gelatinous œdema with hæmorrhage was found covering both thighs, the scrotum and lower part of the abdomen, but no definite changes were found in the internal organs or other parts of the body. The rabbit which received antivenene presented a marked contrast in appearance. A slight degree of scrotal swelling without discoloration was noted at the end of 24 hours and when killed 48 hours after the injection only a limited area of œdema with slight blood staining was present. The dose given to the control animal had caused a serious injury which would probably have had fatal results, while in the serum-treated rabbit the injury was minimal and would easily have been recovered from.

*Coagulant action.*

Employing the technique previously used for testing the coagulant action of venoms (Taylor *et al.*, 1935c) in which venom solutions were added to fresh sheep's

## 276 *Rattlesnake-Mocassin Venoms Compared with Indian Viper Venoms.*

blood the following rates of coagulation were observed with different final dilutions of the venom in blood:—

Venom dilutions ..	1/10,000	1/50,000	1/100,000	1/200,000	1/500,000	1/1,000,000	Saline control.
Coagulation time in mins. and secs ..	0-42	3-45	9-10	13-40	17-35	20-20	29-15

The venom has an active coagulant action in the lower dilutions but is less active than *daboia* or *echis* venoms in the higher dilutions.

### *Neutralization of coagulant action by heterologous antivenene.*

It had been observed (Taylor *et al.*, 1935*a* and *c*) that although the *hæmorrhagin* of viper venoms which had been examined was neutralized by a heterologous antivenene, neutralization of coagulant action was not obtained. A short series of tests of neutralization was carried with dilutions of a mixture of 1 c.c. of unconcentrated *daboia* antivenene and 1 c.c. of a solution of rattlesnake venom containing 1 mg. The final dilutions of venom + serum mixture in sheep's blood and the coagulation times were as follows:—

Venom and serum dilutions. ..	..	1/20,000	1/25,000	1/50,000	Saline control.	Venom control.
Coagulation time in mins. and secs. ..	..	1-50	2-0	2-15	19-6	1-45

The coagulant action of the venom was not neutralized by the heterologous serum and the observation is in accordance with previous findings which indicate the specificity of the coagulant action of viper venoms. Our observations on this and other viper venoms would suggest that the coagulant action of viper venoms is dependent on a specific cytolytic action resulting in the release of thrombokinase.

In the course of experiments carried out in monkeys in which death occurred after a period of delay the blood was found to be incoagulable. As the coagulant action of the rattlesnake venom is similar to that of *daboia* and *echis* venoms the explanation of this finding which we have given (Taylor *et al.*, 1935*c*) for the later venoms, namely, slow defibrination with deposition of fibrin in the vessel walls, will also apply to this venom.

## *ANCISTRODON PISCIVOROUS VENOM.*

### *Neurotoxic action.*

Tests were carried out on pigeons on the same lines as with rattlesnake venom.

Pigeon No. 1.	Dose 0.025 mg.	Survived.
„ No. 2.	Dose 0.05 mg.	Survived.
„ No. 3.	Dose 0.10 mg.	Symptoms of shock similar to those observed with rattlesnake venom. Died in 14 minutes.
„ No. 4.	Dose 0.15 mg.	Similar symptoms. Died in 18 minutes.

The minimal lethal dose is about 0.1 mg. which is one-half of that of the rattlesnake venom, ten times that of *daboia* venom and twice that of *echis* venom. The post-mortem appearances were similar to what have been found in the case of pigeons dying rapidly from the intravenous injection of other viper venoms.

*Neutralization of neurotoxin by daboia antivenene.*

Tests were carried out in pigeons by intravenous injection of mixtures of mocassin venom solution and *daboia* antivenene both in concentrated and unconcentrated form with the following results:—

Venom dose + Antivenene.	Result.
0.2 mg. (= 2 m.l.d.) + 1 c.c. unconcentrated.	Died in 5 minutes.
0.2 mg. (= 2 m.l.d.) + 1 c.c. concentrated.	Died in 5 minutes.
0.1 mg. (= 1 m.l.d.) .. .. .	Died in 20 minutes.

The concentrated antivenene would neutralize at least 4 mg. *daboia* venom by the method of test but does not neutralize a twentieth part of the amount of mocassin venom.

In a similar test with *echis* antivenene 2 c.c. of the serum failed to neutralize 0.2 mg. (=2 m.l.d.) of the mocassin venom, the pigeon dying in 3 minutes. The serum in this case was used in a proportion representing 5 times its homologous titre. The neurotoxin is shown by these tests to be specifically different from that of *daboia* or *echis*.

*Hæmorrhagin content and its neutralization.*

The skin hæmorrhagic dose was ascertained by the intradermal test to be 0.01 mg. The hæmorrhagin content of the venom is thus similar to that of the rattlesnake and *echis* and ten times that of *daboia* venoms.

The hæmorrhagin was neutralized to titre by *daboia* antivenene in exactly the same degree as the hæmorrhagin of rattlesnake venom. It is thus homologous with the hæmorrhagin of the other viper venoms which we have examined.

*Action of mocassin venom on blood coagulation.*

The coagulant action of this venom on sheep's blood in paraffined tubes was tested by the method employed for rattlesnake venom with the following results:—

Venom dilutions ..	1/10,000	1/50,000	1/100,000	1/500,000	1/1,000,000	Saline control.
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Coagulation time ..	No clot at 18 hrs.	28 mins.
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Firm clot at 18 hrs.

## 278 *Rattlesnake-Mocassin Venoms Compared with Indian Viper Venoms.*

A repetition of the test gave similar results and the venom is shown to have a definite anti-coagulant action which we have not observed in any other viper venom we have tested.

An attempt was made to determine whether mocassin venom would neutralize the coagulant action of rattlesnake venom and a series of mixtures of the two venoms were made and added to fresh sheep's blood. With proportions up to twenty parts of mocassin venom to one part of rattlesnake venom no appreciable prolongation of the coagulation time was observed.

The action of mocassin venom solution was also tested on solutions of fibrinogen to which thrombin had been added. In a series of 5 tubes a mixture consisting of 5 drops fibrinogen and 2 drops of thrombin solution was placed and the following additions were made to successive tubes, the results being noted after incubation at 37°C. overnight :—

No. 1.	1 drop of 1/1,000 mocassin venom	..	..	no clot.
No. 2.	2 drops of 1/1,000 „ „	..	..	no clot.
No. 3.	1 drop of 1/1,000 <i>daboia</i> „	..	..	clot.
No. 4.	2 drops of horse serum	..	..	clot.
No. 5.	2 drops of saline solution	..	..	clot.

The anti-thrombic action of mocassin venom is further shown by these findings.

The results of these tests of rattlesnake and mocassin venoms in comparison with the venoms of the *daboia* and *echis* are summarized in the following Table :—

TABLE.

Venom.	Lethal dose for pigeons, mg.	Neurotoxin.	Skin hæmorrhagin dose, mg.	Nature of hæmorrhagin.	Coagulant action.
<i>V. russellii</i> ..	0·01	Specific.	0·10	Homologous for all four venoms.	Specific.
<i>E. carinata</i> ..	0·05	„	0·01		„
<i>C. adamanteus</i> ..	0·20	„	0·01		„
<i>A. piscivorus</i> ..	0·10	„	0·01		Anti-coagulant.

The specificity of the neurotoxic action of the rattlesnake and mocassin venoms has only been determined in relation to the Indian snake venoms which themselves differ specifically in their action. The relationship between the neurotoxins of rattlesnake and mocassin venoms was not determined as no homologous anti-sera were available.

### SUMMARY.

The results of a study of the neurotoxic, hæmorrhagic and blood-coagulant actions of the venoms of the American vipers *Crotalus adamanteus* and *Ancistrodon*

*piscivorous* in comparison with the action of the venoms of the Indian vipers *V. russellii* and *E. carinata* is reported.

The two American viper venoms have a high *hæmorrhagin* content similar to that of *echis* venom and ten times that of *V. russellii* venom. The *hæmorrhagin* of all four venoms is of the same nature and is neutralized to titre by *daboia* antivenene. The *neurotoxin* content of the *rattlesnake* venom is one-twentieth of that of *daboia* venom and one-quarter of that of *echis* venom. The *neurotoxin* content of *mocassin* venom is one-tenth of that of *daboia* venom and half of that of *echis* venom. The *neurotoxins* of the *rattlesnake* and *mocassin* venoms differ specifically from those of *daboia* and *echis* and are not neutralized by large excess of anti-sera prepared against these venoms.

The *rattlesnake* venom has definite coagulant action on blood but is much less active in high dilutions than *daboia* or *echis* venom. Its coagulant action is not neutralized by *daboia* antivenene. *Mocassin* venom has an anti-coagulant action on blood.

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## STUDIES ON INDIAN SNAKE VENOMS.

### Part II.

#### COBRA VENOM: ITS CHEMICAL COMPOSITION, PROTEIN FRACTIONS AND THEIR PHYSIOLOGICAL ACTIONS.

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A STUDY of the chemical composition and location of the toxic principles of cobra venom was made on the same lines as the present authors had employed in the case of daboia venom (Ganguly and Malkana, 1936).

#### CHEMICAL ANALYSIS OF THE INDIAN COBRA VENOM.

The preliminary examination showed the presence of the following elements : C, H, N, S, O and P. Certain inorganic substances and yellow pigments to which the colour of the dried venom is due were also present.

The venom gave the usual reactions for protein and the identification of the proteins was undertaken. The proteins present were found to be (1) coagulable proteids consisting of albumin and globulin, (2) non-coagulable proteids consisting of primary and secondary proteoses only. As phosphorus has been found in this venom detailed examination was made for phospho-proteins, but none were present.

Ether extraction of the dried venom resulted in removal of a dark brown viscid substance which was identified as a mixture of lecithin and cholesterol.

In addition to the free lecithin which was extractable with ether, a part of it was found to be present in combination with the venom proteins in the form of lecitho-proteins.

The total nitrogen content of the venom as estimated by a modified micro-Kjeldahl method, the details of which have been described in the previous paper (*loc. cit.*), was found in successive tests to be on the average 15.06 per cent. Out of this total, however, the nitrogen content of the protein fraction as obtained by precipitation with trichloroacetic acid or sodium-tungstate was ascertained to be 14.01 per cent, representing 87.56 per cent ( $N \times 6.25$ ) of total protein. The non-protein nitrogen as determined from the supernatant fluid left after precipitation of the proteins from the venom solution was estimated to be 1.05 per cent.

#### DETERMINATION OF THE PROTEIN FRACTIONS OF THE VENOM.

The more complex composition of this venom as compared with that of the *daboia*, necessitated the employment of methods different in principle from those employed previously, for the fractionation of the constituent proteins. The methods devised for the present purpose are described below :—

*Estimation of proteoses.*—One per cent venom solution in saline (15 c.c.) was heated to 95°C. on a water-bath for about twenty minutes, till the coagulable proteids were completely coagulated. The coagulum was filtered off and washed and the clear filtrate, representing the primary and the secondary proteoses together with non-protein nitrogenous substances, was treated with an equal volume of fifty per cent trichloroacetic acid to precipitate the proteoses from the rest of the contaminating substances. The precipitate was filtered, washed with 25 per cent trichloroacetic acid and then dissolved in the smallest amount of caustic soda necessary to bring it into solution. It was then neutralized with acetic acid and the volume brought back to the original (15 c.c.) by the addition of distilled water.

The nitrogen content of 2 c.c. portions of the solution thus obtained, representing the amount of primary and secondary proteoses present in 20 mg. of the dried venom, was estimated in successive tests. It was found to contain an average of 4.5 per cent of nitrogen. This is equivalent to 28.12 per cent ( $N \times 6.25$ ) of the two proteoses in the dried venom.

*Secondary protease.*—The remaining solution containing the two proteoses was saturated with sodium chloride, and left in the refrigerator overnight. The following morning the precipitated primary protease which settled down was centrifuged off and the supernatant fluid containing the secondary protease alone was separated and 2 c.c. portions of this were taken separately for determination of the nitrogen content. An average of three readings showed that it contained 2.69 per cent of nitrogen in relation to the dried venom. This represents 16.81 per cent ( $N \times 6.25$ ) of the secondary protease in the dried venom.

*Primary protease.*—The amount of primary protease present was calculated by subtracting the amount of secondary as determined in the previous experiment from the amount of the total proteoses present in the venom. It was thus found to be 11.31 per cent in relation to the dried venom.

*Estimation of the coagulable proteids.*—A fresh sample of the venom solution (5 c.c. of 1 in 100) in saline was heated as described above, for about 20 minutes at

95°C. The coagulable proteids representing the globulin and albumin were coagulated, and the coagulum was filtered and washed to remove any of the adhering non-coagulable proteids and other substances present. The nitrogen content of the coagulum as a whole was estimated in the usual way and was found to be 9.6 per cent in relation to the dried venom, which corresponds to 60.0 per cent ( $N \times 6.25$ ) of the total coagulable proteids.

*Estimation of albumin.*—The globulin and the primary proteose fractions of the venom proteins were precipitated by full saturation of a 1 per cent solution with sodium chloride. The solution was allowed to stand overnight and subsequently the supernatant fluid was separated off either by centrifuging or filtration through a coarse filter-paper. Five c.c. of the clear solution thus obtained was heated at 93°C. for twenty to twenty-five minutes, in order to coagulate completely the rest of the coagulable proteids (the albumin) present in it. The coagulum was filtered, washed with distilled water and its nitrogen content was determined. It was found to contain 6.35 per cent of nitrogen in relation to the dried venom. This represents 39.69 per cent of albumin.

*Estimation of globulin.*—By subtracting the amount of albumin from the total coagulable proteids present in the venom the amount of globulin present was estimated to be 20.31 per cent.

As a further check, however, the nitrogen content of the proteins of the venom precipitable by full saturation with sodium chloride (representing a mixture of globulin and primary proteose) was determined and found to be 4.98 per cent, which represents 31.12 ( $N \times 6.25$ ) per cent of the mixture. Out of this total, the amount of primary proteose which was previously determined to be 11.31 per cent was subtracted, the remainder of 19.8 per cent was, therefore, the amount of globulin present. The figure thus arrived at is slightly lower than the one previously obtained, but since the latter process is more complicated than the former one, the former figure was taken to be more correct.

The composition of the venom was thus found to be :—

				Per cent.
Protein (total)	..	..	..	87.56
Globulin ..	..	..	..	20.31
Albumin ..	..	..	..	39.69
Primary proteose	..	..	..	11.31
Secondary proteose	..	..	..	16.81

along with phospho-lipins (lecithin), cholesterol, some inorganic substances and colouring matter.

#### LOCATION OF THE ACTIVE PRINCIPLE IN THE VENOM FRACTIONS.

The tests of the physiological action of the fractions of the venom were carried out in regard to neurotoxin as indicated by the lethal action in pigeons on intramuscular injection. This is a routine test in use at the Institute and the sample of the venom used had an m.l.d. of 0.4 mg. for a pigeon of 300 g. weight by intramuscular injection. The dose of the venom fractions used is expressed as the equivalent of the original dried venom.

The fraction of the venom as obtained by full saturation with ammonium sulphate, representing the total proteins, was found to contain the full toxicity as judged by the lethal effect in pigeons, the m.l.d. of the protein obtained in this way being the same as the equivalent amount of the dried venom.

The fraction separated by full saturation with sodium chloride or 50 per cent saturation with ammonium sulphate, representing the mixture of the total globulin and primary proteose, was found to have no effect, pigeons surviving a dose up to the equivalent of ten times the m.l.d.

In order to ascertain whether the toxicity could be ascribed to the albumin or the secondary proteose or to both, the latter fraction was separated from the former by heating a 1 per cent solution of the venom (previously freed from the globulin and the primary proteose by full saturation with sodium chloride) to a temperature of six minutes. The albumin fraction was completely coagulated leaving proteose alone in solution. The m.l.d. of this protein fraction calculated the proportion to the original dried venom which it represented showed retained the whole toxicity of the venom. This finding shows conclusively that the albumin fraction is not the toxic one.

As mentioned here that, as in the case of daboia venom, the toxicity of cobra venom is associated with the secondary proteose, but unlike daboia the toxic fraction is to a great extent thermostabile. While heating to 75°C. for six minutes completely destroys the toxicity of the daboia venom, the toxicity of the cobra venom after similar treatment is left unimpaired.

TABLE.

*Showing neurotoxic action of the protein fraction of cobra venom.*

Fractions.	Mean percentage by weight in relation to dried venom.	Intramuscular dose in mg. (equivalent of dried venom) injected in pigeons.	Result.	REMARKS.
1. Total protein ..	87.56	0.4	Died	Full toxicity.
2. Globulin .. ..	20.31	Not tested.	—	—
3. Albumin .. ..	39.69	0.4, 0.6, 0.8, 1.0.	Survived.	Non-toxic.
4. Globulin and primary proteose.	31.12	4.0	„	„
5. Secondary proteose ..	16.81	0.4	Died.	Full toxicity.
6. Primary proteose ..	11.31	Not tested.	—	—

## EFFECT OF HYDROLYSIS ON THE VENOM PROTEOSE.

Having ascertained that the toxicity of the venom resided entirely with the secondary proteose, further experiments were done to see whether the proteose itself or some other agent present in it, either in adsorption or some form of loose chemical combination, was responsible for its physiological action. It was proposed, therefore, to hydrolyse the secondary proteose fraction of the venom to the corresponding inactive amino-acids and then to make a study of the effect of injection of the resulting hydrolysis product in pigeons.

Since a rapid hydrolysis of the venom proteose by the usual methods of refluxing with acid or alkali involved the risk of detoxicating the proteose in the process, due either to the effect of heat or the presence of acids or alkalis, a slow process of hydrolysis by the proteolytic digestion with trypsin was employed. This process involved no risk of damaging any foreign substances in the venom proteose of non-protein origin—the action of trypsin being a selective one, namely, the hydrolysis of proteins to their higher degradation products.

Trypsin was selected as the digestive enzyme for this particular substance because, although its optimum of reaction is reached at pH 8·5, it was found to act quite satisfactorily in digesting albumins and other proteins in neutral media. Moreover, its proteolytic action was found to be much more pronounced than any of the other enzymes such as pepsin or papain under similar circumstances.

The results of control experiments done on the proteolytic action of these three enzymes on a solution of egg albumin (20 c.c.) are shown below :—

Enzyme.	Amount (in c.c.) of 0·0192 N caustic soda required for the formol titration after an incubation period of 24 hours.
1. Papain ..	27·8
2. Trypsin ..	53·6
3. Pepsin ..	25·6

*Hydrolysis of the venom proteose.*—A 1 per cent solution of the secondary proteose of the venom (10 c.c.), measured in relation to the weight of the dried venom, was mixed with trypsin solution (1 c.c.) and to the mixture was added normal saline (9 c.c.) to make a concentration of the proteose in the reaction mixture equivalent to 5 mg. of the dried venom per c.c. The reaction mixture was then incubated at 37°C. for a couple of months to ensure complete hydrolysis. The completeness of the hydrolysis was indicated by the reaction mixture giving a negative biuret test for the proteoses and also by the failure of an equal volume of 50 per cent trichloroacetic acid to cause precipitation of any of the unchanged proteose present.

The amount of proteose hydrolysed in the reaction mixture was also estimated quantitatively by determining the amount of nitrogen transformed into the amino-acid form by the proteolytic hydrolysis of the venom proteose. The estimation was done according to Sørensen's (1907) formol titration method which consisted

in fixing the free amino-group with formalin and then titrating the amount of resulting acidity against a standard alkali at the beginning and the end of the process. The difference between the two readings represents the amount of excess of acidity caused by the setting free of the carboxyl group of the resulting amino-acid and is taken to be a measure of the latter. The results of formol titration of 5 c.c. of the reaction mixture (representing an equivalent of 25 mg. of the dried venom) are as given below :—

Amount (in c.c.) of 0.0194 N caustic soda required in time <i>t</i> .		Difference.
<i>t</i> = 0 hours	<i>t</i> = 2 months	
0 > 7.95 7.95	0 > 10.5 10.5	2.55 c.c.

This represents 0.686 mg. or 2.7 per cent of nitrogen in relation to the dried venom. As has been previously stated, the nitrogen content of the dried venom in the secondary proteose form is on the average 2.69 per cent. Thus the secondary proteose was quantitatively hydrolysed in the process of tryptic digestion to the simple amino-acids.

#### EFFECT OF INJECTION IN PIGEONS OF THE HYDROLYSATE.

The resulting hydrolysis product in doses equivalent in mg. of the dried venom were injected intramuscularly in pigeons. It was observed that the pigeons survived even a dose of 6 mg., which is equivalent to 15 m.l.d's. This clearly indicates that the toxicity of the venom is due entirely to the secondary proteose and is lost when the proteose molecule is broken down to the simpler amino-acids.

#### SUMMARY AND CONCLUSION.

1. The chemical analysis of cobra venom shows that it contains C, H, N, S, O and P.
2. The dried venom contains 87.56 per cent protein, lecithin and cholesterol. The lecithin is present both in the free state as extractable with ether and in combination as lecitho-proteids.
3. The protein fractions are : globulin 20.31 per cent, albumin 39.69 per cent, primary proteose 11.31 per cent and secondary proteose 16.81 per cent.
4. The toxicity of the venom is entirely due to the secondary proteose and is lost on hydrolysing the proteose to the corresponding amino-acids by tryptic digestion.

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## STUDIES ON INDIAN SNAKE VENOMS.

### Part III.

#### ENZYMES IN DABOIA AND COBRA VENOMS.

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THE results of an investigation into the chemical composition and location of the active principles of daboia and cobra venoms have been given in previous papers (Ganguly and Malkana, 1936*a* and *b*). The present paper deals with the nature of the enzymes present in these venoms. Little detailed study has previously been made on the enzymes of snake venoms. Dunn (1934) has demonstrated certain enzymes in the venom of *Crotalus adamanteus* and, amongst others, Potick (1930) has shown evidence of reduction of the phospholipoid contents of the plasma possibly due to the action of snake venoms. Houssay (1930) has also described the presence of enzymes.

The presence of phosphodiastase was also noticed by Rousseau (1934) in cobra venom.

#### IDENTIFICATION OF THE ENZYMES.

##### *I. Proteolytic enzymes.*

The presence of these enzymes, as indicated by their power of splitting up proteins into degradation products such as amino-acids, was investigated in the case of both cobra and daboia venoms. The substrates used for this work were (a) gelatin, (b) crystallized egg albumin, (c) casein and (d) fibrin.

*Action on gelatin and egg albumin.*—In studying the action of venom enzymes on these two proteins, solutions of suitable strength (approximately 2 per cent) of these substances were made in distilled water. Measured volumes (40 c.c.) of each of the solutions were mixed separately with measured volumes (4 c.c.) of 1 per cent of each of these venom solutions. Experiments showed that this amount of venom solution, representing 40 mg. of dried venom, was most convenient for the purpose; a lesser amount caused a very faint hydrolysis which was difficult to measure, whereas the use of too much venom was always avoided owing to paucity of materials.

Each of the reaction mixtures was then allowed to stand in an incubator at 37°C., and the amount of amino-nitrogen formed during the course of proteolytic



hydrolysis was measured immediately after the addition of the venom and at intervals of 24 and 72 hours, samples of 10 c.c. of each of the reaction mixtures being removed for this purpose. Controls were carried out on the same substrates with trypsin, pepsin and papain.

The tests were carried out by (1) Sørensen's formol titration method and (2) by precipitation with trichloroacetic acid.

(1) *Sørensen's formol titration method.*—The ordinary procedure of arresting the alkalinity of amino-groups of the amino-acids with commercial formalin and subsequently titrating with standard alkali (0.0192 N caustic soda) the acidity of the carboxyl groups was employed, and the differences between the initial reading and the subsequent readings were taken as indicating the amount of amino-acid formed in course of that particular period of reaction.

(2) *Precipitation with trichloroacetic acid.*—This consisted in precipitating the undigested proteins of the reaction mixture with 20 per cent trichloroacetic acid according to the method adopted by Dunn (*loc. cit.*) and determining subsequently the amount of non-protein nitrogen in the filtrate. All nitrogen estimations were done by a modified micro-Kjeldahl method as described in a previous communication (Ganguly and Malkana, 1936a).

The results of a series of tests on gelatin and crystallized egg albumin are given in Table I. It will be seen that both cobra and daboia venoms show a digestive action on gelatin and egg albumin more or less comparable with that of pepsin or papain but less active than that of trypsin. The cobra venom showed an action somewhat more marked than that of daboia venom.

TABLE I.

*Showing proteolytic action of daboia and cobra venoms on egg albumin and gelatin as compared with that of known enzymes.*

Protein.	Proteolytic agent.	AMOUNT (IN C.C.) OF 0.0192 N CAUSTIC SODA REQUIRED FOR FORMOL TITRATION PER 100 C.C. OF THE REACTION MIXTURE IN TIME = $t$ .			Amino-nitrogen in mg. per 100 c.c. of the reaction mixture after digestion for 72 hours, determined by trichloroacetic acid method.
		$t=0$ hours.	$t=24$ hours.	$t=72$ hours.	
Gelatin	1. Trypsin ..	89.5	324.5	389.5	80.65
	2. Pepsin ..	97.0	164.0	197.4	26.95
	3. Papain ..	97.0	105.5	132.0	9.40
	4. Cobra venom ..	95.5	107.5	124.0	7.25
	5. Daboia venom ..	95.0	104.0	112.5	4.20
Egg albumin.	1. Trypsin ..	106.0	268.0	290.5	47.2
	2. Pepsin ..	119.5	129.0	134.5	4.2
	3. Papain ..	119.5	139.4	149.5	8.4
	4. Cobra venom ..	117.6	134.5	143.5	9.6
	5. Daboia venom ..	117.0	121.5	129.5	3.4

The presence of proteolytic enzymes in both of these venoms is demonstrated.

*Action on casein.*—A 2 per cent solution of casein was made in an approximately decinormal solution of caustic soda. A measured volume of the casein solution (40 c.c.) thus made was mixed separately with 4 c.c. each of daboia and cobra venom solutions (1 per cent) and incubated at 37°C. for a known length of time. A control experiment using trypsin as the digestive enzyme was made simultaneously under similar conditions. The course of the proteolytic digestion was measured in exactly the same way as in the case of gelatin and egg albumin with the slight modification that the formol titration of the reaction mixture was made against 0.02 N sulphuric acid, and the reduction in alkalinity during the period of incubation was determined and taken as the measure of the amount of amino-nitrogen liberated during the process. The results obtained are given in Table II:—

TABLE II.

*Showing proteolytic action of venoms on casein as compared with the action of trypsin on the protein.*

Protein.	Proteolytic agent.	AMOUNT (IN C.C.) OF 0.02 N SULPHURIC ACID REQUIRED FOR FORMOL TITRATION PER 100 C.C. OF THE REACTION MIXTURE IN TIME = $t$ .		Amount of amino-nitrogen in mg. formed per 100 c.c. after 72 hours (as determined by the trichloroacetic acid method).
		$t=0$ hours.	$t=72$ hours.	
Casein	1. Trypsin ..	418.0	335.0	23.24
	2. Cobra venom ..	166.25	132.75	9.38
	3. Daboia venom	162.50	135.50	7.56

The results indicate that both of the venoms show action on casein in an alkaline medium. The action of cobra venom is in this case again more pronounced than that of the daboia venom.

*Action on fibrin.*—Fibrin prepared from sheep's blood was washed thoroughly with saline solution to remove completely the adhering red blood cells. It was then kept immersed for 24 hours in 0.5 per cent slightly ammoniacal carmine solution in water. The resulting carmin-fibrin was taken out, washed thoroughly to free it from the superfluous dye and was preserved in glycerine for further use.

Small amounts of carmin-fibrin were placed in sugar tubes, to which were added known amounts of venom solutions. The tubes were then incubated at 40°C. for known periods and the effect was observed. Controls of carmin-fibrin in saline and pepsin were also incubated under similar conditions. By the proteolytic action of the enzymes fibrin is digested with the consequent coloration of the supernatant fluid from the liberation of the dye. Absence of colour in the supernatant fluid indicated a negative reaction, the colour of the resulting fluid being

directly proportional to the enzyme activity. The following are the results of a series of tests :—

Proteolytic agent.		Results.	Incubation period.
1. Pepsin	(2 c.c. of 1 in 100)	++++	6 hours.
2. Cobra venom (	do. )	++++	„
3. Daboia venom (	do. )	+++	„
4. Saline control (	2 c.c. )	-----	„

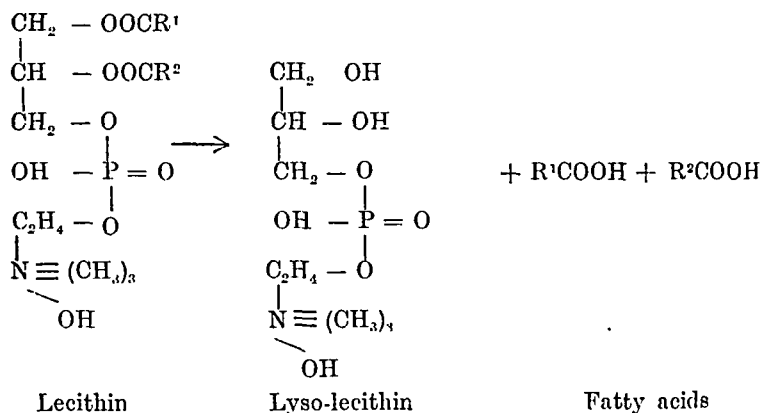
A repeat of this test on another sample using less venom after incubation for about 4 hours gave the following results :—

1. Pepsin	(2 c.c. of 1 in 1,000)	++++
2. Cobra venom (	do. )	++
3. Daboia venom (	do. )	+++
4. Saline control (	2 c.c. )	-----

The results would show the ability of these venoms to digest fibrin and confirm the findings on other substrates.

## II. Lecithinase.

This enzyme possesses the specific property of hydrolysing lecithin into the corresponding lyso-lecithin, liberating in the process the constituent unsaturated fatty acids of the lecithin molecule according to the following scheme (Cameron, 1933) :—



The action of lecithinase on the lecithin molecule is thus quite different from the action of the usual hydrolytic substances on it, which result in the formation of glycerol, phosphoric acid, choline and fatty acids.

This enzyme activity was, therefore, demonstrated by determining the amount of excess of standard alkali necessary to neutralize the acid set free from the lecithin molecule during the course of the reaction.

The substrate employed for this test consisted of a lecithin emulsion in water made according to the following procedure :—

Commercial lecithin (1.5 g.) was dissolved in 2 c.c. to 3 c.c. of methyl alcohol. To the solution 100 c.c. of water was gradually added with continuous stirring, to make a uniform emulsion. The alcohol was then driven off by heating the emulsion

for a couple of hours on a water-bath, after which more water was gradually added to it with stirring to make the volume 100 c.c. again. It was then preserved with a little toluene for further use.

Measured volumes of lecithin emulsion (59 c.c.) were mixed separately with 1 c.c. of 1 per cent daboia and cobra venom solutions, and the reaction mixtures were then incubated at 37°C. Immediately after the addition of the venoms and at intervals of 4 and 24 hours, 10 c.c. samples from each of the reaction mixtures were pipetted out, mixed with 30 c.c. of absolute alcohol and titrated separately against 0.0188 N caustic soda solution. The results of titration are given below :—

Venom.	THE AMOUNT (IN C.C.) OF ALKALI REQUIRED AFTER A REACTION PERIOD = $t$ .		
	$t = 0$ hours.	$t = 4$ hours.	$t = 24$ hours.
Cobra venom ..	7.5	8.25	8.55
Daboia venom ..	7.6	7.80	7.95

A repeat of this experiment, using stronger doses of venoms in the proportion of 2 c.c. of 1 per cent venom solutions per 25 c.c. of lecithin emulsion, gave the following results :—

Venom.	AMOUNT (IN C.C.) OF ALKALI REQUIRED AFTER A REACTION PERIOD = $t$ .	
	$t = 0$ hours.	$t = 24$ hours.
Cobra venom ..	7.70	8.95
Daboia venom ..	7.85	8.80

These results indicate that both venoms contain a lecithin splitting enzyme, its action being of a lesser degree in the case of daboia venom.

### III. Rennetic enzyme.

The power of curdling milk by converting casein into an insoluble para-casein was tested.

Whole milk (30 c.c.) was mixed with 1 c.c. of one per cent calcium chloride solution and portions of 1.5 c.c. of this calcified milk were mixed in sugar tubes with 0.5 c.c. of cobra and daboia venom solutions of different strengths. These were then incubated at 40°C., and the time in minutes and seconds required to cause a curdling of the milk was recorded. With daboia venom there was no clotting, while the results obtained with the cobra venom are given below :—

Venom dilution ..	1/100	1/250	1/500	1/750	1/1,000
Coagulation time in mins. and secs. ..	0-55	1-23	5-15	6-20	14

The above figures are the averages of several readings. There was no firm clotting in any case but simply a curdling of the milk. The results as shown above indicate that, while the cobra venom possesses a marked rennetic activity, no such action is evinced by the daboia venom.

*Action of homologous antivenenes on the enzymes present in the venoms, in vitro.*

In carrying out the tests on the effect of antivenene on the action of the venom enzymes, Kasauli concentrated antivenene (of a titre 1 c.c. of 1 in 4 dilution neutralizing 1 mg. of daboia and 0.5 mg. of cobra venom in standard pigeons) was used.

(a) *Effect on the proteolytic action of the venoms.*—In testing the action of antivenene on the proteolytic enzymes present in the venoms 0.5 c.c. portions of a bivalent cobra and daboia antivenene raised from horses were placed in different sugar tubes with varying proportions of the venoms dissolved in 0.5 c.c. of saline. To each of these tubes a little carmin-fibrin was added and the proteolytic action of these mixtures on fibrin was examined according to the method previously described. Control experiments with the venom solutions, saline and antivenene were made simultaneously under similar conditions. The antivenene was found to have no neutralizing action on the proteolytic actions of the venoms, but on the contrary the mixtures of venom-antivenene had a much more pronounced action than the venom alone. Moreover, it was observed that the antivenene itself had a marked proteolytic effect on fibrin.

Attempts were also made to neutralize the proteolytic action of daboia venom by means of unconcentrated antivenene raised in goats against daboia venom protease. In this case as well it was observed that this antivenene also had proteolytic activity though to a lesser extent than that of antivenene raised from horses. The results of these experiments are given below in Table III :—

TABLE III.

*Showing the result of tests of neutralization of venoms by antivenene.*

	Proteolytic action.	RESULTS.
1. Cobra venom alone ..	++	..
2. Daboia venom alone ..	++	..
3. Cobra venom neutralized with bivalent antivenene.	+++	No neutralization.
4. Daboia venom neutralized with bivalent antivenene.	+++	do.
5. Daboia venom neutralized with anti-daboia goat serum.	++	do.
6. Bivalent antivenene alone (from horse).	+++	Proteolytic.
7. Goat's serum .. ..	++	do.
8. Saline (control) .. ..	--	..

*Effect on rennetic enzyme.*—Neutralization experiments with concentrated antivenene were carried out on similar lines with regard to the rennetic enzymes present in the cobra venom. Varying amounts of venom in 0·5 c.c. of distilled water were mixed with different proportions of antivenene (by volume). The volumes of each of the mixtures were then made up to 1 c.c. by the addition of more water. Half c.c. portions of these neutralized venom samples were mixed separately with 1·5 c.c. portions of calcified milk in different sugar tubes and the time required for curdling of the milk was noticed. All the tubes were kept at a constant temperature of 40°C. The results obtained are given in Table IV :—

TABLE IV.

*Showing the results of tests of neutralization of the rennetic activity of cobra venom by antivenene.*

Amount of venom in mg.	Amount of antivenene in c.c.	Curdling time. Mins. Secs.
2·5	0·5	No curdling.
5·0	0·5	do.
5·0	0·25	do.
5·0	0·15	21-45
5·0	0·125	5-40
5·0	0·10	3-35
5·0	0·05	2-30

The above results show that the rennetic activity of cobra venom can be neutralized by the corresponding antivenene. One c.c. of antivenene was thus found to be capable of neutralizing the rennetic action of 20 mg. of the venom.

## SUMMARY.

1. Both cobra and daboia venoms contain proteolytic enzymes capable of digesting (a) gelatin, (b) crystallized egg albumin, (c) casein and (d) fibrin.
2. Both these venoms contain a lecithin splitting enzyme, the action being of a lesser degree in the case of daboia venom.

3. A rennetic enzyme has been observed to be present in cobra venom but not in daboia venom.

4. The proteolytic action of the venoms cannot be neutralized with antivenene.

5. The rennetic action of cobra venom is neutralizable by antivenene. One c.c. of Kasauli bivalent antivenene (of a titre 1 c.c. of 1 in 4 dilution neutralizing 0.5 mg. of cobra venom in standard pigeons) neutralizes the rennetic activity of 20 mg. of cobra venom.

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## BIONOMICS OF *P. ARGENTIPES*.

### Part I.

#### THE DURATION OF LIFE IN NATURE.

BY

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THE duration of life in nature of the sandfly *Phlebotomus argentipes* was studied in connection with its rôle as the vector of *Leishmania donovani*.

For the transmission of *L. donovani* by the bite of the sandfly, it is necessary that the sandfly should live for at least six days after an infecting blood meal. By the sixth day, or by the time the sandfly is ready for its third feed—since these midges feed every third day—flagellates are usually found to have invaded the pharyngeal and buccal cavities.

That the age and maturity of a flagellate may also influence its infectivity is a point which, though not decided yet, should not be neglected.

Transmission by the bite of the sandfly has been obtained on four occasions in hamsters.

Adler and Theodor (1927) are inclined to think that flagellates of *L. tropica* developing in *P. papatasi* had to be over seven days old before they became infective. On the other hand, Shortt, Sinton and Swaminath (1935) were able to produce an oriental sore in a monkey with flagellates from a *P. sergenti* five days after it was infected. With flagellates of *L. donovani* from *P. argentipes* Shortt, Barraud and Craighead (1927) produced an infection in a mouse by the intraperitoneal



inoculation of flagellates from three sandflies, which had infecting feeds from seven to twelve days previously. No positive results were obtained by the injection of flagellates from flies at the first and second feeds.

The duration of life of these midges under laboratory conditions has been very kindly worked out for us by Dr. K. C. K. E. Raja, whose report is added in the Appendix. No record was kept of the mortality of the flies after the first and second feeds, but it would not be incorrect to estimate that very nearly 50 per cent of flies die about the time of the first oviposition, i.e., the 3rd or 4th day, and 50 per cent of the remainder about the time of the second oviposition. The conception of the life-history of these midges under natural conditions was based on the knowledge of their life in the laboratory, combined with observations on their breeding and feeding habits and general distribution in nature. It was assumed, therefore, that owing to their delicate structure, reluctance to fly any distance when disturbed, tendency to remain in dark and sheltered corners of cattle sheds and living rooms, and to breed in close proximity to such situations, these midges did not voluntarily fly far from their breeding grounds, and that a given population would feed and shelter in that site closest to their breeding grounds which afforded such requirements.

In three preliminary experiments done in 1933, of 105 wild flies marked by the dusting of very fine powdered fluorescein or eosine on them, and released in the same cattle shed where they were collected, one fly was re-captured on the 5th day after release. The poor results were attributed to improper marking methods, and as the sandfly season was rapidly terminating, experiments were suspended till the next year.

In 1934 other methods of marking were also tried, viz., the removal under ether of one middle leg of each fly, or the puncturing of one wing membrane with a fine needle.

In the first series of experiments which were done before the advent of the monsoon, a total of 667 *P. argentipes*, both wild and laboratory-bred, were marked and released on four different occasions. Collections were made at irregular intervals, but none before the 3rd day after release. Of 1,049 flies captured for examination, nine marked flies were found. Of these, 1 was found on the 3rd day, 2 on the 4th day, 4 on the 7th day and 2 on the 15th day after release. The numbers recovered were very small and quite contrary to our expectations.

To obtain some idea, therefore, of what was occurring in nature it was decided to liberate marked flies in given situations and observe their prevalence by making daily catches, beginning from the day after release.

Marked flies were released in seven different situations which included cattle sheds and living rooms, selected because sandflies were naturally present in all of them but one, which will be referred to later.

Marking was done either by the dusting of powdered fluorescein on them, or the puncture under light etherization of one wing membrane. Except in two instances the flies used were laboratory-bred ones, which were marked and released the same day that they emerged from their pupal cases.

To mark the flies with fluorescein a small quantity of the finely powdered dye was placed in a test-tube with the flies; by inverting the tube a few times a fine coating of the powder was laid on the flies. A few from each batch seemed to be

adversely affected by this procedure—they were not as active as they should be—these were excluded from the numbers released. Flies marked like this were fed under laboratory conditions, and it was noted that there was no appreciable difference in the numbers which fed or in their mortality during the subsequent three days, compared with unmarked flies. Some of these flies were tested for the presence of the dye after the first oviposition. Of 31 flies so tested 30 gave the reaction. The use of the powdered dye had this advantage, that by its use we were able to determine one of the natural enemies of sandflies.

To puncture the wing membrane of the flies, they were lightly etherized, and a fine hole made about the middle of the wing with a sharp needle. Judged by the feeding experiments in the laboratory this procedure had no adverse effect on the flies. This method of marking though it was not practised would have been more useful if it was necessary to re-mark captured flies, by making a second puncture on the same wing or marking the unpunctured one.

The results obtained in these experiments are shown in Table I as well as the situations where the flies were released, method of marking used and the day of collection. Excluding experiment No. 14 where regular catches were not made—the collectors being refused admission because one of the cows had recently calved—on 17 consecutive occasions when marked flies were released in a shed and collections made regularly, in one instance only was a fairly large number of flies recovered, 13·3 per cent of those liberated. This was the first occasion when flies were released in a dwelling house and only 30 flies were used. In the other 16 experiments the recovery rate varied from 0 out of 95 to 8·7 per cent of 80 flies released. The total recovery rate for all experiments together was 3·6 per cent.

The number of marked flies recovered was always small and the different marking methods made no appreciable difference in the recovery rate.

Of the marked flies recovered 79 were caught on the 1st day after release, 18 on the 2nd, 9 on the 3rd, 7 on the 4th, 2 on the 5th and 3 on the 6th day. After the 6th day no marked flies were found though collections were continued for several days on many occasions.

Earlier in the year, before the break of the monsoon as previously mentioned, marked flies were recovered up to the 15th day after release. The experiment was therefore repeated to observe what numbers were recoverable if no collection was done for about ten days after the liberation of marked flies. Of 428 flies released not a single one was recovered in collections made on the 9th, 12th, 16th and 19th day. Two hundred and twenty-eight of these flies were marked by wing puncture and 200 with fluorescein (*see* Table Ia).

It might here be mentioned that for this work the same three men were employed who were especially trained in the collection of these midges for periods varying from 4 to 10 years. The field work was supervised by one of the first two authors (S. M. or R. O. A. S.) once or twice a week.

It is hardly conceivable that but 3 to 4 per cent of flies present in any given situation are collected after a careful search with a torch. That a fairly large proportion of flies are missed by our methods is evident when the figures in Table II are considered.

In Table II will be found the totals of the daily collection of flies from certain sheds where the experiments were carried out. The flies have been classified according to the state of the blood meal present. Since these midges do not feed again till they have completed the digestion of the previous blood and oviposited, and as this process takes approximately three days, a rough idea of the age of the fly could be obtained. Those with a full blood meal, half or more digested meal, and fully gravid without the visible presence of blood, corresponding in age to one day, two days and three days' old flies respectively.

The proportion in which these flies were found was 56, 15 and 29 per cent respectively.

A possible explanation for these findings is that the recently fed ones were in the majority as they were collected before they found suitable hiding places to remain undisturbed, while the meal was being digested. Those of the middle category were smallest as they were already in hiding in cracks and holes in the wall from where they had no reason to move. The gravid ones were found in larger numbers probably because they were on the move again as their time for oviposition was approaching.

On the assumption, therefore, that only such flies as were in hiding were missed, it will be seen that we were recovering about 56 per cent of the flies present in a given place.

To determine whether any of the flies migrated from situations where they were released, in experiments Nos. 2 and 4, Table I, collections were made from the cattle shed where they were released as well as the dwelling house in the vicinity. This shed and house was surrounded by a thick belt of vegetation and had no other houses within a radius of 50 yards. Five hundred flies were released in this shed on two occasions and only one fly and that too a male was recovered from the dwelling house.

The observation that the proportion of gravid flies collected from any site is in keeping with the numbers of the younger flies, the fact that breeding grounds were often found close to or inside the cattle sheds and living rooms, and also that any positive evidence of migration was not forthcoming, led us to infer that the previous assumption regarding the movement of these flies was correct, and that they did not migrate to any extent. The question of their fate, therefore, naturally arose. If they were not present where they were released and not found in the surrounding houses, they must either be in hiding in places unknown to us, or they were being destroyed.

The natural enemies of these midges were therefore examined. In the stomach and gut contents of lizards it was possible to detect the typical wings of *P. argentipes*; also in examining some spiders captured in these situations, reactions suspicious of fluorescein were obtained. To verify this observation about 1,152 flies were marked and released in a shed from which only the spiders were captured for examination. Of 20 spiders examined 6 gave the test for fluorescein, showing that they were responsible for the destruction of a good number of these midges. The spiders most active in this direction were predaceous ones, and belonged to two genera. One species of the Genus *heteropoda*, and the other belonged to the family of the jumping spiders, the *Attidæ*. The web-spinning species are rather a protection to these

midges, as they take shelter on or under the webs, without getting entangled in the meshes.

These experiments were all done with laboratory-bred flies, and one experiment was therefore done with wild flies, to observe if any different results would be obtained. In experiment No. 2 (see Table Ia) 100 *P. argentipes*, which were collected mostly in one shed, were released in the same shed after they were marked with fluorescein. Among them were a few *P. papatasi*. No count was made of the latter, but it was estimated that there were not more than 10 or 12. On making daily collections from this shed only two marked flies were recovered, and both were *P. papatasi*.

In view of this result, in the last experiment done this year, *P. papatasi* were used, to determine their disposal as compared with *P. argentipes*. From four houses, three situated fairly close together and the fourth a little apart, all the *P. papatasi* and *P. argentipes* were collected, marked with fluorescein and liberated in the isolated house. Sixty-three *P. papatasi* and 21 *P. argentipes* were released, and 15 *P. papatasi* and 1 *P. argentipes* were recaptured. No marked flies were found in the other three houses which were also searched each day. In this experiment, therefore, nearly 24 per cent of *P. papatasi* were recovered. This is a high proportion of recoveries compared with 4·7 per cent of *P. argentipes* in this or 3·6, the average for the previous 18 experiments; or even 13·3 per cent, the best recovery rate in any single experiment with *P. argentipes*.

*P. papatasi* are far more active in their habits compared with *P. argentipes*, and it is probable that the larger proportion of recoveries was due to the fact that they were better able to elude their natural enemies. Dr. Raja's note on this finding is appended.

In the Graph overleaf are shown the numbers of flies collected daily from two sheds, as well as the irregular collections from three other sheds. It will be seen that the natural destruction prevailing coupled with the daily collection of flies, caused no marked diminution in the numbers present. That the introduction of a large number of marked flies made no appreciable difference in the numbers collected the next day is also evident.

Experiments Nos. 10 and 12 shown in Table I were conducted in a dwelling house where sandflies were very scarce, about one or two at most being found each week. The release of 75 and 100 flies on two occasions resulted in the recapture of 3 marked ones. The total number of flies collected on 16 occasions was 14. Breeding grounds were located round this house in two instances.

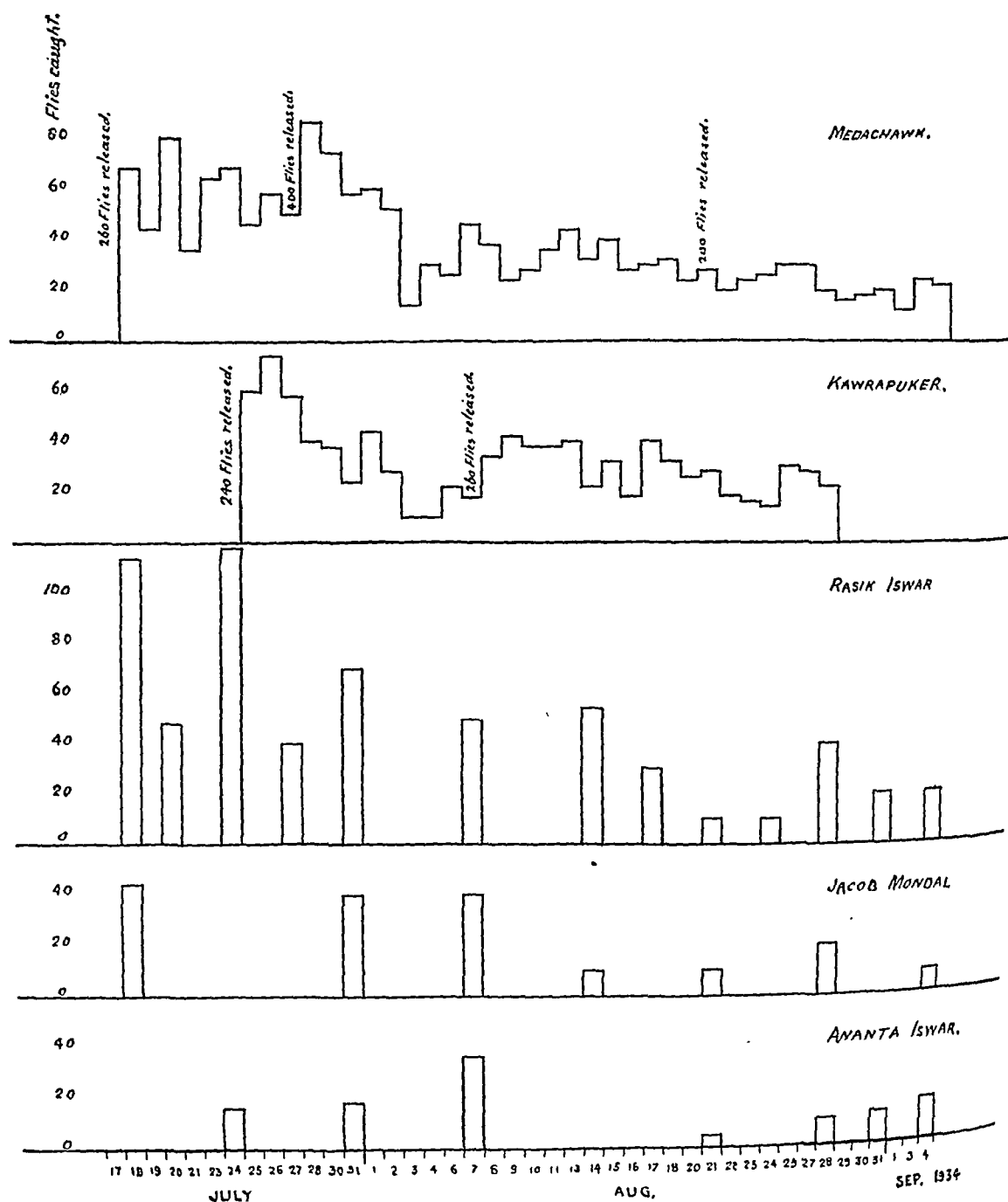
We do not feel justified in drawing any definite conclusions from these observations. They are, nevertheless, very suggestive that a considerable destruction of these midges occurs under natural conditions and among their natural enemies; spiders, of two predaceous species, are responsible to a great extent for the diminution in their numbers.

As these experiments were done in an inter-epidemic period, and only three flies were recovered on the sixth day, the findings are not inconsistent with the incidence of kala-azar at the present time in this area, assuming *P. argentipes* to be the vector. If these findings could be confirmed one reason for the slow spread

of the disease could be explained ; it would also indicate that under natural conditions the inoculation of flagellates into a victim by a large number of infected sand-flies is improbable.

## GRAPH.

Showing number of *P. argentipes* collected daily and periodically in five cattle sheds.



The question naturally arises whether one of the factors responsible for an epidemic of kala-azar is an alteration of balance between the insect vectors and their natural enemies. In the laboratory a single spider of the *Attilid* family has been known to consume as many as 30 sandflies in a day. Spiders are not as prolific breeders as many species of insects. It is only after a series of moults, usually about nine in number, that they attain full maturity. Any adverse factor, such as a drought, about the time of their breeding season, which as far as we have been able to determine occurs about twice a year, may result in a considerable diminution of the young members by depriving them of small insects on which they are solely dependent for food. Their cannibalistic tendency also will be more in operation during such a period and cause a still further decrease in their numbers. As many of these species are purely domestic, their value in reducing the numbers of blood-sucking insects which enter dwelling houses is deserving of further investigation.

Our thanks are due to Dr. K. C. K. E. Raja for his help with the figures, to Dr. F. H. Graveley for very kindly identifying the spiders, and to the insect collectors for their co-operation in this work.

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Situation.	Date.	Method of marking.	Number of flies released.	Number of flies captured and marked.	Day of									
					1	2	3	4	5	6	7	8	9	10
1 Medachawk cattle shed.	17-7-34	Fluorescein	260	{ Captured .. Marked ..	68 4	41 0	80 1	36 0	.. ..	64 1	60 0	48 0	52 0	12 0
2 Kawrapuker cattle shed.	24-7-34	"	240	{ Captured .. Marked ..	68 5	79 1	67 0	40 0	.. ..	36 0	24 0	44 0	5 0	5 0
3 Medachawk cattle shed.	27-7-34	"	400	{ Captured .. Marked ..	87 5	.. ..	74 0	58 1	61 1	53 0	15 0	31 0	11 0	11 0
4 Kawrapuker cattle shed.	7-8-34	"	260	{ Captured .. Marked ..	39 10	42 2	38 3	39 0	.. ..	41 1	23 0	33 0	11 0	11 0
5 Medachawk cattle shed.	21-8-34	"	200	{ Captured .. Marked ..	21 2	24 0	26 0	30 0	.. ..	30 0	19 0	17 0	14 0	14 0
6 Trivango Mondol house.	29-8-34	"	30	{ Captured .. Marked ..	20 3	13 1	15 0	13 0	.. ..	13 0	13 0	.. ..	.. ..	.. ..
7 Trivango Mondol house.	8-9-34	"	75	{ Captured .. Marked ..	17 3	.. ..	14 1	14 0	13 0	11 0	14 0	6 0	6 0	6 0
8 Kawrapuker cattle shed.	4-9-34	"	150	{ Captured .. Marked ..	37 2	22 0	23 1	13 2	.. ..	12 0	17 0	.. ..	.. ..	.. ..
9 Kawrapuker cattle shed.	11-9-34	"	80	{ Captured .. Marked ..	21 3	16 4	13 0	16 0	.. ..	.. ..	10 0	.. ..	.. ..	.. ..
10 Phiren Patro house.	7-9-34	"	75	{ Captured .. Marked ..	1 1	.. ..	0 ..	2 0	0 ..	.. ..	0 ..	.. ..	.. ..	.. ..
11 Medachawk cattle shed.	14-9-34	"	180	{ Captured .. Marked ..	27 8	.. ..	.. ..	27 2	.. ..	17 1	10 0	15 0	15 0	15 0
Carried over ..			1,950	Recovered ..	46	8	6	5	1	3	0	0	0	0

COLLECTION.																Total caught.	Total of marked flies.	Percentage re-covered.	REMARKS.	
10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25					
53	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	}	519	6	2.3	..
0	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..					
10	10	..	22	18	..	..	..	..	..	..	..	..	..	..	..	}	424	6	2.5	..
0	0	..	0	0	..	..	..	..	..	..	..	..	..	..	..					
6	47	38	23	28	36	..	45	33	41	28	29	33	..	25	27	}	838	7	1.7	..
0	0	0	0	0	0	..	0	0	0	0	0	0	..	0	0					
1	33	..	26	27	18	17	14	30	..	28	23	..	..	..	..	}	531	16	6.1	..
0	0	..	0	0	0	0	0	0	..	0	0	..	..	..	..					
10	12	..	24	22	..	..	..	..	..	..	..	..	..	..	..	}	264	2	1.0	..
0	0	..	0	0	..	..	..	..	..	..	..	..	..	..	..					
8	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	}	102	4	13.3	..
0	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..					
..	19	..	..	8	..	..	..	..	..	..	..	..	..	..	..	}	116	4	5.3	..
..	0	..	..	0	..	..	..	..	..	..	..	..	..	..	..					
..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	}	124	5	3.3	..
..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..					
..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	}	76	7	8.7	..
..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..					
..	1	..	..	1	..	..	..	..	..	..	..	..	..	..	..	}	5	1	1.3	..
..	0	..	..	0	..	..	..	..	..	..	..	..	..	..	..					
..	27	30	12	14	19	..	..	..	..	..	..	..	..	..	..	}	181	11	6.1	..
..	0	0	0	0	0	..	..	..	..	..	..	..	..	..	..					
0	0	0	0	0	0	0	0	0	0	0	0	0	0	..	0	3,180	69			



Situation.	Date.	Method of marking.	Number of flies released.	Number of flies captured and marked.	DATE							
					1	2	3	4	5	6	7	8
Brought forward ..			1,950		46	8	6	5	1	3	0	0
12	Phiren Patro house.	25-9-34	Fluorescein	100	{ Captured ..	7	1	0	0	0	0	0
					{ Marked ..	2	0	..	..	..	..	..
13	Irivango Mondol house.	25-9-34	"	100	{ Captured ..	11	9	5	9	..	3	3
					{ Marked ..	5	0	0	0	..	0	0
14	Bonhugli cattle shed.	11-8-34	"	120	{ Captured ..	..	41	..	37	..	..	..
					{ Marked ..	..	0	..	0	..	..	..
15	Bonhugli cattle shed.	29-9-34	Wing puncture.	95	{ Captured ..	25	13	25	28	..	..	28
					{ Marked ..	0	0	0	0	..	..	0
16	Bonhugli cattle shed.	10-9-34	{ Wing puncture 140 Fluorescein 150	{ 290	{ Captured ..	26	48	35	23	33	..	21
					{ Marked {	*5	*2	..	*1	..	..	..
						†1	†3	†1	..	0	..	0
17	Bonhugli cattle shed.	23-9-34	{ Wing puncture 213 Fluorescein 100	{ 313	{ Captured ..	56	47	18	28	21	..	14
					{ Marked {	*7	*5	*2	0	0	..	0
						†2	..	..	..	..	..	..
18	Bonhugli cattle shed.	4-10-34	{ Wing puncture 165 Fluorescein 138	{ 303	{ Captured ..	52	..	28	30	18	27	..
					{ Marked {	*5	..	0	*1	*1	0	..
						†6	..	..	..	..	..	..
TOTAL RELEASED ..			3,271	Recovered	79	18	9	7	2	3	..	..

—concl'd.

LECTION.															Total caught.	Total of marked flies.	Per- centage re- covered.	REMARKS.	
11	12	13	14	15	16	17	18	19	20	21	22	23	24	25					
0	0	0	0	0	0	0	0	0	0	0	0	..	0	0	3,180	69			
0	0	0	..	..	..	..	..	..	..	..	..	..	..	..	}	9	2	2.0	..
..	..	..	..	..	..	..	..	..	..	..	..	..	..	..					
2	..	.	..	..	..	..	..	..	..	..	..	..	..	..	}	57	5	5.0	..
0	..	..	..	..	..	..	..	..	..	..	..	..	.	.					
..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	}	78	0	..	..
..	..	..	..	..	..	..	..	..	..	..	..	..	..	..					
22	35	..	27	..	..	..	..	..	..	..	..	..	..	..	}	295	0	..	..
0	0	..	0	..	..	..	..	..	..	..	..	..	..	..					
10	16	..	..	..	..	..	..	..	..	..	..	..	..	..	}	285	13	4.5	Wing punctu 8 = 5.7 per ce Fluorescein 5 = per cent.
..	..	..	..	..	..	..	..	..	..	..	..	..	..	..					
0	0	..	..	..	..	..	..	..	..	..	..	..	..	..	}	250	16	5.1	Wing punctu 14 = 6.5 per ce Fluorescein 2 = per cent.
..	..	..	..	..	..	..	..	..	..	..	..	..	..	..					
..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	}	155	13	4.2	Wing punctu 7 = 4.2 per ce Fluorescein 6 = per cent.
..	..	..	..	..	..	..	..	..	..	..	..	..	..	..					
0	0	0	0	0	0	0	0	0	0	0	0	0	..	0	4,309	118	3.6	..	

uncture.  
in.

Situation.		Date.	Method of marking.	Number of flies released.	Number of flies captured and marked.							
						1	2	3	4	5	6	7
1	Medachawk cattle shed.	29-9-34	Wing puncture 228 Fluorescein 200	428	Captured .. Marked ..	..	..	..	..	..	..	..
2	New cattle shed	9-11-34	Fluorescein	<i>P. argentipes</i> 100 <i>P. papatasi</i> ? 10	Captured .. Marked ..	30	..	43	45	31	33	14
3	Paran Bagh ..	17-9-35	Fluorescein	<i>P. argentipes</i> 21 <i>P. papatasi</i> 63	Captured .. Marked {	37	30	23	..	..	34	..
4	Other houses close to Paran Bagh.	17-9-35		..	Captured .. Marked ..	47	29	21	..	..	24	0

SECTION.																Total caught.	Total of marked flies.	Percentage re- covered.	REMARKS.
11	12	13	14	15	16	17	18	19	20	21	22	23	24	25					
..	8	..	..	..	13	..	..	13	..	..	..	..	..	..	}	62	0	..	..
..	0	..	..	..	0	..	..	0	..	..	..	..	..	..					
9	8	..	7	..	..	..	..	..	..	..	..	..	..	..	}	262	2	..	Both <i>P. papatasii</i> ? 20 per cent re- covered. No <i>P.</i> <i>argentipes</i> recovered.
0	0	..	0	..	..	..	..	..	..	..	..	..	..	..					
..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	<i>P. papatasii</i> 15	15	23·8	}	..
..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	<i>P. argen- tipes</i> 1	1	4·7		
..	..	..	..	..	..	..	..	..	..	..	..	..	..	..					
..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	}	..	0	..	..
..	..	..	..	..	..	..	..	..	..	..	..	..	..	..					

TABLE II.

Situation.		Full blood meal.	Half or more digested.	Gravid.	Total flies.	Full blood meal.	Half or more digested.	Gravid.	Unfed females.	Males.
Medachawk	..	298	41	93	519	298	41	93	7	80
Kawrapuker	..	57	23	57	446	57	23	57	95	214
Medachawk	..	353	84	203	805	353	84	203	19	146
Kawrapuker	..	166	37	113	508	166	37	113	37	155
Medachawk	..	128	37	67	264	128	37	67	3	29
Trivango ..	..	41	26	18	102	41	26	18	1	16
Trivango ..	..	44	17	18	116	44	17	18	1	36
Kawrapuker	..	32	18	10	124	32	18	10	12	52
Kawrapuker	..	23	3	8	76	23	3	8	4	38
Medachawk	..	71	26	38	181	71	26	38	4	42
Trivango ..	..	24	7	7	57	24	7	7	1	18
TOTALS ..		1,237	319	632	3,198	1,237	319	632	184	826
Percentages ..		56.5	14.5	28.8	..	38.6	10.0	19.7	5.7	25.8

## APPENDIX.

### *Duration of life of the sandfly P. argentipes under laboratory conditions and other statistical data.*

BY

K. C. K. E. RAJA.

IN order to form an idea of the longevity of sandflies under laboratory conditions, the following data are available. During the period May 1933 to September 1934, 12,998 laboratory-bred flies took a first feed, and of them 3,337 survived till the third feed. Of these 3,337 flies, 236 were known positives, and records were kept of their order of dying out. Dr. Smith considers that the presence of flagellates in the fly does not in any way shorten its life-history. If the rates of mortality of this small sample of 236 flies is applied to the larger group of 3,337 flies, and if in view of the smallness of the number, the rates are graduated by a third order parabola, before being applied to the larger population (3,337) we obtain the figures shown in the following Table as representing the population of flies at the beginning of each age period given (see also Graph) :—

TABLE.

*The ages shown are based on average experience of the numbers of days corresponding to the respective feeds (as given to me by Dr. Smith), in relation to which the enumerations of flies took place.*

Age in days.	Population of flies.	Rate of mortality.	Number of deaths.
6.5 to 9.0 ..	3,337	0.41441	1,383
9.0 to 11.5 ..	1,954	0.55100	1,077
11.5 to 14.0 ..	877	0.55193	484
14.0 to 16.5 ..	393	0.50348	198
16.5 to 19.0 ..	195	0.49193	96
19.0 to 21.5 ..	99	0.60356	60
21.5 to 24.0 ..	39	0.92465	36
24.0 to 26.5 ..	3	1.54148	3

We started with 12,998 first fed flies. The first feed usually takes place within 24 hours after emergence from the pupal case. No record was kept of the number of flies which survived for the second feed between the 3rd and 4th day after emergence. The third feed corresponds roughly to 6·5 days and subsequent feeds take place, Dr. Smith considers, approximately at intervals of two days and a half. The following Table gives the percentage of flies that died out within the respective periods indicated, when we started with a population of 12,998 flies :—

Within the first		Percentage that died.
6·5 days	..	74·33
9·0	..	84·97
11·5	..	93·25
14·0	..	96·98
16·5	..	98·50
19·0	..	99·24
21·5	..	99·70
24·0	..	99·98

A fly becomes infective only about the time of the third feed and it may be seen that by that time (6·5 days) only a little over 25 per cent survive. The bearing that this fact may have on the transmission of kala-azar is dealt with elsewhere by Drs. Smith and Mukerjee.

#### *Capture of marked flies.*

An examination of the figures relating to recovery of marked flies in Table I reveals the fact that the proportions recovered show a significant difference in the case of some experiments when compared in pairs and that significance emerges when the series of 20 experiments is considered as a whole. But the numbers recovered were always small except in one instance. In all the experiments together 3,271 flies were released and 118 recovered. It has been stated by Drs. Smith and Mukerjee that two species of predaceous spiders, natural enemies of the sandfly, were responsible for producing a certain amount of depopulation among them.

These experiments relate to *P. argentipes* which is known to be less active in its habits than *P. papatasii*. It is possible that the sluggish habits of the former render it more easily a prey to its natural enemies than the latter. An experiment on exactly the same lines carried out with *P. papatasii* gave 15 recoveries when 63 flies were released. Unfortunately, as the Inquiry was closing, further experiments with this species could not be continued. However, a comparison of the rates of recovery in the two cases based on the available data shows that the difference between the rates is significant.

Proportion caught among	<i>P. argentipes</i>	$\frac{118}{3,271}$	= 0.0361.
"	"	<i>P. papatasii</i>	$\frac{15}{63}$ = 0.2381.
Difference	..	..	.. 0.2020.

If we start with the assumption that both (3,271 and 63) are random samples from the same population and that the difference between the two rates may be due solely to chance, our best estimate of probability of recovery is

$$p = \frac{118 + 15}{3,271 + 63} = 0.0399 \quad q = 0.9601.$$

The standard error of difference = 0.0249.

$$\frac{\text{difference}}{\text{st. error}} \quad .. \quad 8.11.$$

Hence it is quite significant.

It may be that *P. argentipes* being less active than *P. papatasii*, the natural enemies were able to destroy the former more easily than the latter. If active habits of the fly interfered with the process of catching, the ratio caught in the case of *P. papatasii* should have been significantly less than in the case of *P. argentipes*. This is not so.





## THE TRANSMISSION OF *L. DONOVANI* BY THE BITE OF THE SANDFLY *P. ARGENTIPES*.

BY

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[Received for publication, 16th March, 1936.]

THE object of this note is to place on record one more successful transmission of *L. donovani* to a hamster (*Cricetulus griseus*) by the bite of the sandfly *P. argentipes*. The hamster which showed the infection was one of 16 which were given an average of ten known positive feeds each, before they were separated off to complete the incubation period of approximately 16 months.

In another series of 20 hamsters, each hamster was given one known positive feed at the 5th, 6th, 7th and 8th feed of an infected fly. No positive results were obtained in this series.

The multiplication and disposition of the flagellates of *L. donovani* in the sandfly *P. argentipes* being so suggestive of transmission by the 'bite' of the fly, no factor other than host-resistance was cited as the cause of failure in the earlier transmission experiments. The fact that three successful transmissions to hamsters had already been obtained (Shortt, Smith, Swaminath and Krishnan, 1931; Napier, Smith and Krishnan, 1933) was evidence that flagellates capable of causing an infection were inoculated at the bite of an infected sandfly, if not always, then on certain occasions. That the incubation period for the development of the infection was so prolonged—16 months in the hamster after being 'bitten' by infected sandflies—might have been due as well to the smallness of the dose as to a resistance in the victim.

In the search for some factor in the hamster which might have raised its resistance to a small dose of flagellates, it was found that hamsters kept in the laboratory for long periods on a diet of soaked gram and vegetables—either lettuce

TABLE.  
*Showing transmission experiments in hamsters.*

HAMSTERS WHICH HAD EIGHT OR MORE KNOWN POSITIVE FEEDS.				HAMSTERS WHICH HAD 4 TO 7 KNOWN POSITIVE FEEDS.				HAMSTERS WHICH HAD UNDER FOUR KNOWN POSITIVE FEEDS.			
Serial number.	DIED.		SACRIFICED.	DIED.		SACRIFICED.	Serial number.	DIED.		SACRIFICED.	Serial number.
	Incubation period in months.	Result.		Incubation period in months.	Result.	Incubation period in months.		Incubation period in months.	Result.	Incubation period in months.	
2	..	..	Positive	17	..	17	4	..	..	16	Negative.
6	..	..	Negative	16	..	16	19	13	Negative	..	..
7	17	Negative	..	..	..	17	25	..	..	11	Negative.
8	..	..	Negative	15	Negative	..	..	..	..	..	..
9	..	..	..	21	..	..	..	..	..	..	..

10	..	..	20	"	..	..	..	..	..	..	..
11	..	..	20	"	..	..	..	..	..	..	..
12	..	..	19	"	..	..	..	..	..	..	..
13	14	Negative	..	..	..	..	..	..	..	..	..
14	8	"	..	..	..	..	..	..	..	..	..
15	4	"	..	..	..	..	..	..	..	..	..
16	3	"	..	..	..	..	..	..	..	..	..
18	..	..	16	Negative	..	..	..	..	..	..	..
20	..	..	14	"	..	..	..	..	..	..	..
21	..	..	13	"	..	..	..	..	..	..	..
22	..	..	13	"	..	..	..	..	..	..	..

or cabbage—had, when compared with hamsters recently imported from China, a higher adrenal content, when assayed biologically. It was the opinion of the Director, the late Colonel Acton, that an infection with *Leishmania* was not compatible with a high adrenal content. In addition to the low blood pressure and the pigmentation of kala-azar patients being ascribed to adrenal deficiency, he had not seen a case of visceral leishmaniasis in a victim either of diabetes or leucoderma, two diseases fairly common in Bengal, where kala-azar is endemic, and which are often associated with hyper-adrenalæmia.

The only variation made in these transmission experiments, therefore, was in the care and feeding of the animals, which were given a liberal mixed diet and plenty of water. That the general health of the hamsters was influenced by this treatment was reflected by the success obtained in breeding these animals for the first time under laboratory conditions. The results obtained in the attempts to transmit the infection to them were, however, disappointing.

The susceptibility of the hamster to *Leishmania* being so great, it is improbable that the host resistance factor was operative in these or on the previous occasions when this animal was used. Unless the rôle of the sandfly in the transmission of kala-azar be excluded—a procedure which is not justified in view of four successful transmissions secured up to date, as well as the close relation between this insect and the disease, both epidemiologically and biologically—the cause of failure to transmit the infection should be investigated in the sandfly, with special reference to the presence and virulence of the flagellates in the buccal cavities of infected flies, on which the success or otherwise of an infection depends. Better results would possibly be obtained if the laboratory technique was modified, so that more natural methods of breeding and feeding *P. argentipes* were practiced.

The results of the transmission experiments are given in the above Table.

Our thanks are due to Colonel R. N. Chopra and to Dr. J. C. Gupta for very kindly estimating the adrenal content of the hamsters, to Insect Collectors Messrs John A. Day and P. Roy for much work in connection with the feeding of the infected flies on the hamsters.

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## FURTHER OBSERVATIONS ON INDIAN RELAPSING FEVER.

### Part III.

#### PERSISTENCE OF SPIROCHÆTES IN THE BLOOD AND ORGANS OF INFECTED ANIMALS.

BY

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THE persistence of spirochætes in the blood and organs of infected animals at times other than the recognized attacks has attracted considerable attention, because it introduces problems of the greatest interest and importance connected with immunity and the possibility of cure.

The presence of spirochætes in the blood between the attacks of fever has been noted by many authors (Serjant and Foley, 1914 ; Moselli, 1923 ; and other workers). Persistence for varying periods after the final attack has also been shown repeatedly. Weichbrodt (1920), Todd (1920) and Mayer (1922) have proved the blood to be infective at periods of 42, 32 and 22 days after the last attack respectively. More recently special significance has been attached to the persistence of the organisms in various internal organs, but particularly the brain, and a considerable literature has accumulated around this subject.

Such resistance has been much discussed in regard to its relationship to questions of immunity, resistance to re-infection or super-infection, and the possibility of cure with arsenical preparations.

Ehrlich and Hata (1910), in their early experiments with mice, considered that a daily negative examination of the blood for sixty days, followed by successful re-infection, established the proof of cure. Inability to re-infect demonstrated a latent infection (*immunitas non-sterilans*).

Various authors (Brault and Montpellier, 1914; Plaut and Steiner, 1920; Weichbrodt, *loc. cit.*; Werner, 1924) have noted the presence of spirochaetes in the cerebro-spinal fluid of patients suffering from the disease, and the last named found that the organism persisted long after all fever had ceased, and also after repeated doses of arsenicals. The observations of Kudicke, Feldt and Collier (1924) are of special importance in this connection. Strains isolated by them from the cerebro-spinal fluid at different periods differ from each other and always from those found in the blood.

Buschke and Kroo (1923) first claimed that relapsing-fever spirochaetes may invade the brain substance in mice and drew attention to the significance of such infections. Numerous observers have corroborated these findings. *S. recurrentis*, *duttoni* (including its variants), *hispanica*, and a Manchurian strain have all been incriminated. Such infections may last for long periods [as long as 409 days (Beunders and van Thiel, 1932)] after the blood is free of spirochaetes. The frequency with which these different strains produce brain infections differs very considerably, while individual strains may also show considerable variation in this respect at different times. It would appear from the literature, however, that strains transmitted by the tick are more likely to invade the brain than *S. recurrentis*, although this is not without exception, for Coleman (1934) reports the absence of brain infections so far as the Californian strain investigated by him is concerned.

The significance of these residual brain infections has received much attention. Buschke and Kroo (*loc. cit.*) and Kroo (1926) claimed that the spirochaetes in this situation gained special characteristics, i.e., developed a special neurotropism, that immunity to further infection lasted only as long as the organisms remained in the brain and that they persisted in this situation in spite of treatment. According to Rosenholtz, Owsjannikowa and Treflow (1928) the spirochaetes enter the nervous tissue and live in a kind of symbiotic relation with this tissue (neurobiose). Levaditi and Anderson (1928) believe that such organisms persist in an invisible stage, although Beunders and van Thiel (*loc. cit.*) deny the existence of such a phase. More recently, Tokura (1933) denies the invasion of the brain tissue and considers that the organisms persist in the blood islands of the smaller capillaries resulting from thrombus formation. The existence of a special neurotropism is denied by Prigge (1926) and Prigge and Rothermundt (1928*a* and *b*), who hold that residual infection is the result of a low degree of immunity developed in the host against infection associated with an organism of low virulence. Hiroki (1933) and Toda and Hiroki (1934) oppose this view, however, as they have been able to show that the number of residual infections does not depend upon the virulence of the strains used for experiment.

The relation of residual infections to the continued state of immunity is of considerable importance. Buschke and Kroo (*loc. cit.*) and Kroo (*loc. cit.*) supported the view of *immunitas non-sterilans* originally held by Ehrlich and Hata. Heronimus (1928) and Jackimow (1929) held that immunity to re-infection was due in the first place to the persistence of the brain infection assisted to a varying extent by the development of humoral antibodies. Prigge (*loc. cit.*) and Prigge and Rothermundt (*loc. cit.*) gave pride of place to antibody formation and held that after natural recovery with satisfactory production of antibodies, residual brain infections were rare.

Most observers agree that once spirochætes are established in the brain they resist the action of salvarsan or similar drugs to a greater extent than spirochætes in other situations, and that under such conditions the brain is either incapable of sterilization (Schauder, 1928; and other workers) or is the last organ to be cleared before cure (Schureus and Weisbecker, 1926). Opinion is divided, however, on the power of the drug to prevent residual infections in the first place. Those who favour a special neurotropism for these parasites consider that residual infections occur in spite of treatment (Kroo, *loc. cit.*; Lebedjeva and Ssinjuschina, 1927; Schauder, *loc. cit.*). The opposing school (Tomioka, 1924; Reiter, 1925; Prigge, *loc. cit.*; Johannessohn, 1926) holds that early and energetic treatment will completely sterilize the blood and prevent residual infections even though they may occur when treatment has been delayed or when inadequate. It is difficult to compose the views of the numerous observers on this subject, but the variation in the number of brain infections occurring even with the same strain at different times, which has prompted Rothermundt (1932) to question the value of such experiments as a criterion of the efficacy of any therapeutic substance, has undoubtedly much to do with the differences in the views expressed.

Apart from the work of Kudicke, Feldt and Collier (*loc. cit.*), little or no consideration has been given to the capacity of these organisms for rapid serological variation and its possible bearing on the problems under discussion. This omission seems to us to be fundamental and it is with the object of investigating these aspects of the problem that our experiments were undertaken.

Our investigation has followed the lines of those of other observers, in that we have attempted to prove the presence or absence of spirochætes in the tissues at various periods during and after infection by sub-inoculating a series of animals from the organs of the infected animal and noting the presence or absence of infection in the sub-passages. For the purposes of this investigation the sub-inoculations were carried out on different days corresponding to the periods between the first and second attacks, i.e., the '1st interval', and at various times, early and late, after the second attack, i.e., the '2nd interval'. In different cases sub-inoculations were made from one or more of the following tissues: peripheral blood, heart blood, liver, spleen, brain, and bone-marrow. In the case of the organs not less than half of the emulsified organ was distributed between two to four fresh animals. The sub-inoculations were made intraperitoneally and the blood of the sub-passage was examined under the dark-ground illumination for the following fifteen days. Certain of the animals died within this period from the effects of the inoculations, but, as a rule, there were one or more survivors which enabled conclusions to be drawn for each group.

In addition, the types of spirochæte found were identified and the kinds of antibody present in the blood at the various phases of the disease were estimated where necessary both in the parent squirrels and the sub-passages from them.

Our results have been arranged in four tables. Table I shows the results of sub-inoculations carried out at different times during the '1st interval', i.e., within seven days after the end of the 'first attack'. The experiment divides itself into two series; the first consisting of three squirrels, in which the peripheral blood and organs were used for sub-inoculation, the second, a group of seven animals, where the heart blood only was sub-passaged during life.





In the first series the peripheral blood was proved infective in one out of the three cases, while the organs were proved positive in two out of the three squirrels used for the experiment. These findings coincide with those of other observers already referred to. The type of spirochæte found differed in each case from that which was responsible for the primary attack, and was obviously capable of developing in the body as the serological observations showed; antibodies to the infecting type alone had been developed so far.

In the second series the heart blood of five out of the seven animals was found infective, although no evidence of infection of the peripheral blood could be found by the ordinary methods of examination (i.e., dark-ground illumination). These results are interesting in that they bear out the views that we have already expressed that the mechanism of infection consists essentially of a series of waves of infection by different types of spirochæte, some developing into visible attacks, others remaining latent, and each wave being overcome by specific antibody formation until the infection finally dies out. During the intervals between the visible attacks the spirochætes appear to retire into the internal organs, including the interior of the heart, where the process of development takes place until sufficiently numerous to overflow into the peripheral circulation.

Although practically all the different types of spirochæte we had isolated were represented in the initial infections, the types found in the sub-passages were limited, with only two exceptions, to the types 'A' and 'B', corroborating our opinion that there is a definite urge on the part of the subsidiary types to return to the primary types when opportunity offers (Cunningham and Fraser, 1934).

The second table (Table II) shows the results of sub-inoculations carried out from the blood (in one case, the peripheral blood, in the remainder, the heart blood) and organs of a series of six squirrels at periods immediately following the second attack (first relapse).

These results are entirely different from those obtained from sub-passages carried out from animals during the '1st interval' in that no evidence of living spirochætes could be found in either the blood or the tissues of the various organs, including the brain. This is explained to a great extent by the development of antibodies in the blood to not less than three types of spirochæte, which has been sufficient to counteract any further multiplication of the organisms.

Tables III and IV give the results of a similar series of experiments carried out at much later periods.

In Table III the peripheral blood of sixteen squirrels has been sub-passaged at intervals varying between 163 and 59 days after the end of the first attack. In Table IV sub-passages from the blood and organs of nine animals have been made between 130 and 57 days after the primary infection.

In no case in either series did infection result, proving the sterility of the blood, or blood and tissues, at the particular periods the sub-inoculations were carried out. Examination of the serum at the date of sub-passage once more showed the presence of antibodies to one or more types of spirochæte, more commonly the latter; the titres, while generally lower than those recorded at the end of the attack, being still sufficient to prevent any recurrence or continuance of infection with these particular types, thus causing the infection to die out.

TABLE II.

Showing the results of sub-inoculation of the blood and organs of infected squirrels at a period immediately following the first relapse.

Squirrel number.	TYPE OF SPIROCHETE IN		Day of 2nd interval sub-inoculation performed.	TYPE OF SPIROCHETE FOUND BY SUB-INOCULATION FROM						REACTION OF SERUM TO SPIROCHETAL TYPES ON DAY OF SUB-INOCULATION.						
	First attack.	Relapse.		Peripheral blood.	Heart blood.	Liver.	Spleen.	Brain.	Bone-marrow.	A.	B.	C.	D.	E.	G.	I.
10517	D	AB	1	:						10,240	5,120	—	5,120	—	—	:
10661	C	B	1	:						2,560	1,280	5,120	—	—	—	:
10436	B	A	4	:		:				10,240	2,560	—	—	40	—	:
10437.	B	A	4	:						20,480	10,240	—	—	40	—	:
10339	B	AE	5	:						10,240	5,120	—	10,240	1,280	10,240	:
988†	A	B	11	—	:					5,120	10,240	1,280	—	—	—	—

TABLE III.

Summarizing the results of sub-inoculation of the peripheral blood of squirrels which have survived an attack of the disease for various periods previous to sub-inoculation.

TABLE III.  
*Showing the results of sub-inoculation of the peripheral blood of squirrels which have survived an attack of the disease for various periods previous to sub-inoculation.*

Squirrel number.	TYPE OF SPIROCHÆTE IN		Number of days since attack.	Sub-inoculation from peripheral blood.	TITRE OF SERUM TO SPIROCHÆTAL TYPES AT TIME OF SUB-INOCULATION.						
	First attack.	Relapse.			A	B	C	D	E	G	I
7779	E	..	163	—	—	—	40	40	—	—	—
8078	B	..	125	—	160	40	320	—	—	—	80
8097	I	..	124	—	—	—	40	—	—	—	40
8338	C	..	103	—	160	40	320	—	—	—	—
8409	E	..	96	—	80	—	—	640	160	—	—
8579	C	..	82	—	—	40	160	—	—	—	—
8574	E	..	82	—	1,280	320	320	—	—	—	20
8588	G	..	81	—	40	10	640	—	—	1,280	—
8590	I	..	81	—	640	320	—	—	—	40	640
8623	C	..	78	—	—	—	1,280	—	—	—	—
9663	G	C	76	—	10	1,280	80	20	—	640	..
8672	C	..	73	—	40	40	1,280	—	—	—	—
9815	E	..	66	—	—	40	160	—	160	—	..
8769	E	..	64	—	640	1,280	40	—	1,280	40	80
8775	I	..	64	—	640	320	80	—	40	—	40
9208	E	..	59	—	—	—	—	—	5,120	—	—

TABLE IV.

Showing the results of sub-inoculation of the organs of squirrels which have survived an attack of the disease for various periods previous to sub-inoculation.

Squirrel number.	TYPE OF SPIROCHÆTE IN		Number of days since last attack.	TYPE OF SPIROCHÆTE FOUND BY SUB-INOCULATION FROM						TITRE OF SERUM TO SPIROCHÆTAL TYPES AT DATE OF SUB-INOCULATION.						
	First attack.	Relapse.		Peripheral blood.	Heart blood.	Liver.	Spleen.	Brain.	Bone-marrow.	A	B	C	D	E	G	I
8441	A	:	130		:					640	—	40	320	—	—	10
9324	G	:	96	:				:	:	—	80	80	20	—	20	:
9103	I	+	95		:					40	40	—	—	—	—	80
9123	E	:	93		:					—	—	—	—	—	—	—
9157	B	:	89		:					—	1,280	—	—	—	—	20
9170	G	:	89		:					—	—	—	—	—	10	—
9177	E	:	88		:					—	—	—	—	80	—	—
9277	A	:	79		:	:				1,280	1,280	320	—	—	—	:
9992	D	:	57	:					:	20	—	—	2,560	—	—	:

## SUMMARY AND CONCLUSIONS.

The conclusions to be drawn from these experiments may be summarized as follows:—

1. Animals infected with *Sp. carteri* maintain spirochætes in the blood and internal organs throughout the interval between the first and second attacks (and would undoubtedly do so between the second and third attacks should a third attack occur). Such latent infections can be demonstrated by sub-inoculation of the blood, particularly the heart blood, or organs taken from an infected animal during this period.
2. On the other hand, 'residual infections', as the term is usually understood, and particularly invasion of brain tissue, do not occur with the Indian louse-borne strain of spirochæte.
3. The spirochæte found during the '1st (or 2nd) interval' by such means always differs in type from the spirochæte initiating the primary attack, and will, in all probability, simulate the type present in the relapse, if a relapse should subsequently occur.
4. The serological examination of the blood carried out at various periods during and subsequent to infection bears out the conclusion previously reached by us that the disease essentially consists 'in most cases of a succession of invasions by different types of spirochæte which vary in intensity and duration; the essential difference between cases which exhibit "single" attacks and those showing "relapses" being only one of degree in that the later attacks are "latent" in the one case and demonstrable by blood examination in the other'.

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*Note.*—References marked \* were consulted in the *Tropical Diseases Bulletin* only and not in the original.

## FURTHER OBSERVATIONS ON INDIAN RELAPSING FEVER.

### Part IV.

#### RE-INFECTIONS AND SUPER-INFECTIONS.

BY

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THE consideration of re-infection and super-infection raises various questions dealing with the immunity acquired by the primary infection. In the first place the degree of protection produced is of primary importance with regard to re-infection and will almost certainly have a definite influence on a super-infection.

A high grade immunity should resist re-infection with the same strain as long as it lasts. A considerable amount of work designed to test the extent of this resistance has been recorded by different observers.

Ross (1911) (Kenya strain) and Nicolle and Blaizot (1913) (Tripolitan strain) showed that monkeys could be re-infected shortly after recovery with a second inoculation of the same strain. Serjent and Foley (1914) showed that spirochætidal substances protected monkeys one and a half months after recovery, but they were absent from the blood in four months. These observers also held that the immunity acquired by an attack in man was not of long duration. Oliver (1924) held the same opinion, as re-infections in certain of his cases occurred six months after recovery. In our earlier work with the Madras strain (Cunningham, 1925)



re-infections were carried out in monkeys at different times after recovery with a varying amount of success.

With regard to super-infection, Kudicke, Feldt and Collier (1924) showed that it was possible to superimpose an infection during the interval notwithstanding the fact that the first infection was still in existence, and Steiner and Steinfeld (1926) studying the effect of repeated inoculations in the same animal showed that with a mild primary infection it is possible to superimpose a more intense infection by re-inoculation.

The conception of an *immunitas non-sterilans* complicates the question still further, for, under these conditions, resistance to further infection is evidence of the continuance of the primary infection not of an acquired immunity. Indeed, Ehrlich and Hata (1910) considered that a successful re-infection after 60 days' negative examination of the blood was a proof of cure.

As already noted, however, the existence of residual brain infections has raised doubts as to the efficacy of this test, and Kitschenski and Brussin (1926) have pointed out that considerable difficulty may be experienced in deciding whether a re-appearance of spirochætes in such circumstances is due to a true re-infection, a super-infection, or a provocation of an already existing infection.

While the specificity of various strains has been fully recognized (Weichbrodt, 1920; Nicolle and Conseil, 1923) we do not think that sufficient weight has been attached to the variations in type which occur within the same strain in the consideration of these problems. We, therefore, believe that the publication of certain experiments which were devised to show the basic importance of these changes in such cases may serve a useful purpose.

### I. RE-INFECTIONS.\*

We have already published a detailed study of the course of antibody formation (Cunningham and Fraser, 1935) in infected animals and a further consideration of this question need not detain us. What is of more importance for our present purpose is the question of the length of time such antibodies remain in the blood stream after the primary infection.

In the first table (Table I) the agglutinin content of the serum of five monkeys infected with different types of spirochætes has been recorded at 30 days' intervals for periods extending over a year, and, in most cases, until the agglutinins have finally disappeared from the blood. One of the animals (No. 181A) reacted positively to the infecting type for 570 days, while another (No. 188C) only reached 360 days, and two other cases (Nos. 186B and 185E) became negative at intervening periods. The fifth case (No. 187D), however, showed a relatively high titre (1:320) on the 480th day and would undoubtedly have continued to do so for a longer period than did the other cases had the animal survived. Antibody produced in response to a relapse disappears sooner (No. 181), and secondary agglutinins formed in the course of single attacks are also of shorter duration.

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\* It must be understood that the serological types of spirochæte referred to in these experiments have been obtained from a single strain of *S. carteri* derived originally from a case of relapsing fever in a human being. For further details as to the methods used in isolating these types, the reader is referred to our previous papers, particularly Part I of this series (*Ind. Jour. Med. Res.*, 22, No. 4, April, 1935).

TABLE I.

*Showing the agglutinin titre of the serum of monkeys infected with various types derived from the same strain of S. carteri taken at intervals of 30 days.*

Monkey number.	Type of spirochaete in		Titre of serum examined at 30 days' intervals.																			
	First attack.	Relapse.																				
			30	60	90	120	150	180	210	240	270	300	330	360	390	420	450	480	510	540	570	600
181	A	B }	A	5,120	1,280	640	640	320	320	320	160	160	160	160	160	160	640	160	80	20	0	0
	B		10,240	320	320	80	40	20	20	20	20	20	20	20	20	10	0	0	0	0	0	0
186	B	— }	B	2,560	640	160	40	80	80	80	80	80	320	160	40	10	20	20	..	..	..	..
	A		1,280	640	80	40	40	80	40	20	20	80	20	10	0	0	0	0	..	..	..	..
188	C	— }	C	10,240	1,280	320	320	80	80	40	40	160	80	80	0	0	..	..	..	..	..	..
	B		320	20	20	0	0	0	0	0	10	10	10	0	0	..	..	..	..	..	..	..
187	D	—	D	10,240	2,560	640	320	320	320	640	640	320	320	1,280	640	320	320	320	..	..	..	..
	E		2,560	640	640	160	20	20	20	20	20	80	20	0	0	10	0	10	..	..	..	..

*Note.*—Each sample of serum was tested to types 'A', 'B', 'C', 'D', 'E', 'G', 'H' and 'I'. Only positive reactions have been recorded in each case.

As far as immunity due to antibody in the blood is concerned, therefore, monkeys infected under these conditions would be incapable of resisting re-infection with the same strain about 19 months after the primary infection and in many cases much earlier.

The success of re-infection carried out at times *when antibody is present in the blood* will depend to a great extent on the size of the infecting dose\*, the type of spirochæte used for the re-infection, and the amount and types of antibody present.

Table II gives details of an experiment designed to show the influence of these various factors on subsequent re-infection. A series of squirrels was selected which had been infected from 64 to 163 days previously. The antibody content of the blood was estimated to each type of spirochæte and then re-infection was attempted with the type which showed the *highest* concentration of antibody. The type selected by this means was generally, but not universally, the type used in the primary infection (*see* Nos. 8078 and 8590). In only one case out of nine (No. 7779) was the re-infection successful, although all the controls gave a positive result. In No. 7779 the titre of the serum was positive only in a 1 : 40 dilution which was obviously too weak to resist the re-infection.

The same squirrels were now subjected to a second re-infection with another type of spirochæte selected on the principle that the blood contained *no* corresponding antibody. The same infecting dose of spirochætes was employed as formerly. On this occasion infection followed in each case. It is obvious, therefore, that a successful re-infection with the same strain, when antibody is present in the blood, depends upon the type of spirochæte used, and the types and concentration of antibody in the circulation: re-infections with 'like' (homologous) types are negative, provided that the antibody content is sufficient; those with 'unlike' (heterologous) types are positive.

The effect of the re-infection on the antibody content is also of some interest. Column 9 of Table II shows the estimation of the agglutinin titre at varying periods after the first re-infection (Nos. 8769, 8775 and 8590) and after the second re-infection (No. 8078). In the former case a considerable enhancement of the previous titre has occurred, while in the latter, agglutinins for the 'heterologous' spirochæte used for the re-infection have also appeared, showing that the re-infection is a new and separate entity and is thus more in the nature of a super-imposed infection.

## II. SUPER-INFECTIONS.

The possibility of super-imposing a fresh infection on one already in existence will, to a great extent, depend upon the same factors. Kitschenski and Brussini (*loc. cit.*) have recorded the difficulty experienced by them in distinguishing between a re-infection, super-infection and the provocation of an existing infection in these cases.

We are of opinion, however, that precise information as to the course of antibody formation and the type of spirochæte used for the various infections will give the clue to a correct interpretation in the majority of cases.

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\* The same infecting dose was employed throughout these experiments. Control squirrels were also inoculated to prove infectivity on each occasion.

TABLE II.

*Showing the results of re-infections carried out with types of spirochetes selected on the basis of the type of antibody present or absent from the blood.*

Squirrel number.	Type of spirochete in first attack.	Number of days after attack.	TITLE OF SERUM AT DATE OF FIRST RE-INFECTION.								Type of spirochete used for first re-infection.	Result.	Type of spirochete used for second re-infection.	Result.	TITLE OF SERUM AFTER RE-INFECTION.								Date of test.
			TITLE OF SERUM AT DATE OF FIRST RE-INFECTION.				TITLE OF SERUM AFTER RE-INFECTION.																
			A	B	C	D	E	G	I	A					B	C	D	E	G	I			
8078	B	125	160	40	320	—	—	—	80	C	—	E	+	160	40	20,480	—	10,240	—	—	10 days after second re-infection.		
8338	C	103	160	40	320	—	—	—	—	C	—	D	+	..	..	died	..	..	..	..	..		
8579	C	82	—	40	160	—	—	—	—	C	—	A	+	..	..	died	..	..	..	..	..		
8623	C	78	—	—	1,280	—	—	—	—	C	—	B	+	..	..	died	..	..	..	..	..		
8769	E	64	640	1,280	40	—	1,280	40	80	E	—	D	+	40	80	40	—	5,120	—	—	13 days after first re-infection.		
8574	E	82	1,280	320	320	—	—	—	20	A	—	died	..	..	..	..	..	..	..	..	..		
8775	I	64	640	320	80	—	40	—	40	A	—	G	+	1,280	40	80	—	20	—	—	13 days after first re-infection.		
7779	E	163	—	—	—	40	40	—	—	D	+	died	..	..	..	..	..	..	..	..	..		
8590	I	81	640	320	—	—	—	40	640	I	—	E	+	160	160	20	—	—	—	5,120	13 days after first re-infection.		

*Note.*—Control squirrels inoculated at the same time as the experimental animals with each type of spirochete used in the experiment were all positive 24 hours after inoculation.

Tables III, IV and V give examples of super-infections carried out at various stages of the disease, namely, during the 'first attack' (Table IV), the '1st interval' (Table III), and the '2nd interval' (Table V).

In each case the result of super-infections with types of spirochæte 'homologous' and 'heterologous' to those responsible for the original infection is given. For the sake of convenience the results obtained with infections super-imposed during the '1st interval' (Table III) are described first.

In each of the four examples given super-infection has been carried out 48 hours after the end of the first attack. In the first two squirrels (Nos. 10186 and 10170) the primary infection was obtained with type 'G', in the third and fourth squirrels (Nos. 10191 and 10184) with type 'E'. In the first and third animals super-infection was attempted with the 'homologous' type, in the second and fourth with a 'heterologous' type ('A' type in No. 10170 and 'B' type in No. 10184). In each case the serum was examined for agglutinins immediately prior to the second inoculation, and also on the day following, and the results, which are also given in the table, show a rising tide of antibody formation to the spirochæte responsible for the primary infection.

The increase in the titre seems to be especially marked after super-infection with the 'homologous' type (squirrels Nos. 10186 and 10191) which seems to have acted as a stimulant in this case.

With regard to the results of the super-infection, the two squirrels inoculated with 'heterologous' types (Nos. 10170 and 10184) showed visible infections on the day following which were proved to be due to the spirochæte responsible for the super-infection. In the case of the other two squirrels, infected with the 'homologous' types, spirochætes appeared in the blood of the first squirrel (No. 10186) on the third day. These were shown to be mixed 'B' and 'C' types. In the other squirrel (No. 10191) spirochætes appeared on the fifth day. These proved to belong mainly to the 'B' type. No infection with the spirochæte responsible for the super-infection occurred in these two cases. The serological tests carried out during the course of the disease thus showed clearly that the second appearance of spirochætes was in the nature of a relapse to the original infection and that the super-infection had failed to take.

Table IV gives an analysis of the effects of super-infection during the 'first attack'. In this series the primary infection was carried out with 'A' type and the super-infection with 'E' type. By this means the course of the two infections could be followed independently of each other.

In the first squirrel (No. 10788) the super-infection was carried out 24 hours after, and in the second squirrel (No. 10776) 48 hours after, the primary infection. In the first case the two infections appeared to run concurrently, the first attack was not prolonged and the titre of the serum to the two organisms on the third day of the first interval was 1/2560 to both types.

A relapse, due to a third type 'D', appeared in due course.

In the second squirrel the general course of the disease was similar to that in the first squirrel. In this case, however, the infection caused by the second inoculation definitely lagged behind the primary attack, spirochætes being present

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*Note.*—The type of spirochaete present in the blood at various stages has been recorded by substituting the letter denoting the type found for the usual plus sign.

*Giving examples of super-infections carried out during the first attack with 'unlike' (heterologous) types of spirochaetes.*

E —

Note.—The type of *aplocheilae* present in the blood at various stages has been recorded by substituting the letter denoting the type found for the usual plus sign.

Giving examples of super-infections carried out during the 2nd interval with 'like' (homologous) and 'unlike' (heterologous) spirochætes.

Serial number.	Squirrel number.	Day of disease →	1	2	3	4	5	6	7	8	9	10	15	20	25	30	35	40
1	10697	Inoculations →	E	+	+	+	+	+	+	+	+	+	↓ Serum E 5120 D 1280	↓ B				
2	10634	Inoculations →	G	+	+	+	+	+	+	+	+	+	+	↓ Serum B 1280 G 5120				
3	10516	Inoculations →	C	+	+	+	+	+	+	+	+	+	+	↓ Serum A 1280 C 2560	↓ B			
4	10701	Inoculations →	A	+	+	+	+	+	+	+	+	+	↓ Serum A 20480 B 640	↓ D	↓ Serum A 2560 B 10240 D 5120	↓ I	↓ Serum A 5120 B 10240 D 10240 I 20	

Note.—The type of spirochæte present in the blood at various stages has been recorded by substituting the letter denoting the type found for the usual plus sign.



in the blood for eight days. A reference to the third squirrel (No. 10660), where the super-infection was delayed until the third day of the primary infection, proves this to be the case, and shows very clearly the true sequence of events.

On the third day, before the introduction of the 'E' type infection, the only type of spirochæte present in the blood was type 'A'. Two days later (i.e., the fifth day of the primary infection and the third day of the super-infection) about  $\frac{1}{4}$  of the spirochætes reacted with the 'A' type, and  $\frac{3}{4}$  with the 'E' type, serum, showing that the primary infection had nearly run its course while the super-infection was still progressing in intensity. The following day only 'E' type spirochætes were found, and, two days later, when the squirrel unfortunately died, serum, taken on the day of death reacted well to 'A' type, but was negative to 'E' type, showing that the latter infection had not progressed sufficiently to produce antibodies, while the primary infection had definitely reached the '1st interval' stage.

In Table V examples of super-infections carried out during the 2nd interval are given. In the first squirrel (No. 10697) the super-infecting type of spirochæte is identical with the type responsible for the primary infection (type 'E'), and, in the second squirrel (No. 10634), with the type found in the relapse (type 'B').

In both cases antibodies to the infecting types were present in the blood to a marked degree, and consequently the super-infection failed to materialize.

The next two squirrels (Nos. 10516 and 10701) give examples of super-infections carried out with types of spirochæte different from those which appeared in the primary infection or its relapse. In each case the super-infection was immediately successful. Indeed, in the fourth case the squirrel went through a completely new infection which included a relapse, the serum taken from the animal immediately before death exhibiting antibodies to all the four types of spirochæte which had appeared in the blood.

It should be noted, however, that the majority of infections carried out during the 2nd interval are in reality 're-infections' rather than 'super-infections' because second relapses in squirrels infected with *S. carteri* are very rare and it has already been shown that the spirochæte does not persist in the blood or organs once the relapse has come to an end.

These tests show that super-infections in this particular form of the disease follow the same rules as re-infections, and, to a great extent, depend for their success or failure upon similar factors. If successful the two infections run independently of each other, the exact form taken depending upon the stage of the disease chosen for the super-infection.

It is perhaps unfortunate that residual infections were not found in the Indian louse-borne form of the disease because we were thus unable to subject cases of this nature to a similar range of tests with the object of comparing the type of spirochæte present in such cases with those responsible for the primary attack, and also of investigating the extent of the *immunitas non-sterilans* claimed under such conditions by the methods illustrated above.

## SUMMARY AND CONCLUSIONS.

Experiments have been carried out with a view to investigating the part played by the different serological types derived from the Indian louse-borne strain of spirochæte in producing 're-infections' and 'super-infections'. The results which have been obtained warrant the following conclusions:—

1. Monkeys infected with the Indian louse-borne strain of spirochæte retain antibodies in their blood for varying periods up to 19 months after the primary infection. With the disappearance of antibody re-infection will always be successful and will not differ in any way from a completely new infection.
2. When antibody is present in the blood the success or failure of re-infection depends upon the size of the infecting dose, the type of spirochæte used for the re-infection and the concentration and types of antibody produced by the primary infection.
3. Under ordinary conditions of dosage re-infection will fail when sufficient antibody to the type of spirochæte used for the re-infection is present in the blood. When antibody to the re-infecting type of spirochæte is absent or insufficient re-infection can readily be obtained.
4. Re-infection, whether carried out with 'homologous' or 'heterologous' types of spirochætes, produces a rapid and considerable increase in the corresponding antibody content of the blood.
5. Super-infections depend for their success or failure upon similar factors.
6. When antibodies derived from the primary infection are circulating in the blood (i.e., at periods subsequent to the first attack) super-infections carried out with ordinary infecting doses are only successful with 'heterologous' types of spirochæte. 'Homologous' types of spirochæte fail to infect under such conditions.
7. A successful super-infection runs its course as a separate infection which can be distinguished from the different stages of the primary infection by appropriate serological tests even when the infection has been super-imposed during the 'first attack'.
8. The clinical appearances which result from a super-infection depend upon the particular stage of the disease chosen for the super-infection.

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## TWO EASY METHODS OF TRANSPLANTING TUBERCLE BACILLI DIRECTLY FROM SOLID TO LIQUID CULTURE MEDIA.

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In the study of the biology of the tubercle bacillus, it is often necessary to observe the character of growth of different strains of this organism on liquid media.

One of the characteristics of this bacillus is that on liquid media its growth occurs on the surface. It is important, therefore, in sowing broth cultures, that the seed used for inoculating should remain floating, for, should it sink, no satisfactory growth would occur.

Successful surface transplantation can easily be accomplished from one broth culture to another by gently lifting with a platinum loop a piece of the surface film from an old culture and lightly depositing it on the surface of fresh broth; but this is not so easy when a strain is sown for the first time on broth from a solid medium.

Perhaps some success may be attained with human tubercle bacilli which often produce on solid media a dry scaly growth which can be removed and lightly laid on the surface of broth on which it remains floating and continues to grow. But this is not always successful, for sometimes the growth sinks and it is more difficult with moist growth such as that of the bovine tubercle bacillus or the slimy growth of the avian tubercle bacillus. Various methods have been tried to overcome this difficulty with more or less success.

Cubes of solid medium with the thickness corresponding to the depth of the fluid have been placed in the medium and sown with cultures, or pieces of cork have been used to support pieces of agar sown with culture on the broth.

Cobbett (1917) recommends the introduction of a small piece of cotton-wool into the broth flask before sterilizing. The wool swells up and extends to the surface and forms a sufficient support for the bacilli which are planted upon it.

Eastwood and recently Lutz (1925) have worked with a combined solid and liquid medium. In this method agar or serum is solidified in a slope in a conical flask and some broth is afterwards added to the bottom; when the surface of the solid medium is planted, the growth gradually extends and creeps over the surface of the broth from which it can be lifted off for subsequent broth cultures.

The methods devised by the writer which have been found very useful and satisfactory are described below:—

The essence of the first method is to keep the seed culture floating on the surface of the broth by placing it on a strip of filter or other absorbent paper and resting the latter on a piece of vaselined paper or other light material. This keeps floating on the broth and supports the filter-paper with the seed culture and growth is favoured by the diffusion of the medium through the filter-paper, the projecting ends of which remain in contact with the medium.

The details are as follows:—

A piece of ordinary paper about 1/10 mm. in thickness and of convenient size, say, about four inches long and two inches broad, is soaked in hot vaseline at a temperature of about 140°C. for about half a minute and drained and the excess of vaseline is wiped off from the surface.

Small pieces, about three-fifths of an inch square, are cut from this and are distributed, one in each, in test-tubes of about three-fourth inch diameter. The exact size of the pieces is of no great consequence provided they are only slightly smaller than the diameter of the test-tube in which they are kept, so that they are capable of being inserted with ease and at the same time are sufficiently large to leave an adequate space between the under-surface of the paper and the wall of the test-tube for a free play of the platinum loop for the purpose of removing the paper (Plate XXIV, fig. 2).

Into the same test-tubes are further placed pieces of ordinary filter-paper, one in each, cut into narrow strips about 1/6 to 1/5 inch wide and of such length that when laid across the vaseline-paper their ends extend about 1/8 to 1/4 inch beyond either edge of the latter. These tubes are sterilized in the autoclave at 15 lb. pressure for one-half hour.

Several such tubes, each containing a piece of vaseline-paper and a strip of filter-paper, may be prepared, sterilized, and kept ready for use whenever required.

The technique is simple. Firstly, by a little manipulation the filter-paper strip is made to lie lengthwise across the centre of the piece of the vaseline-paper in the test-tube without opening it (Plate XXIV, fig. 1). Secondly, with a 5 mm. platinum loop, a drop of sterile broth or salt solution is placed on the filter-paper which makes it stick to the vaseline-paper on which it is lying.

Thirdly, a loopful of culture to be inoculated is removed from the solid medium and is placed in the centre of the filter-paper. A young actively growing culture is always preferable.

Lastly, the whole assemblage, i.e., the vaseline-paper with the filter-paper and the culture, is carefully lifted and removed from the tube with the aid of the same platinum loop inserted under the vaseline-paper and is laid on the surface of the

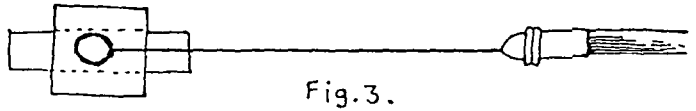


Fig. 3.

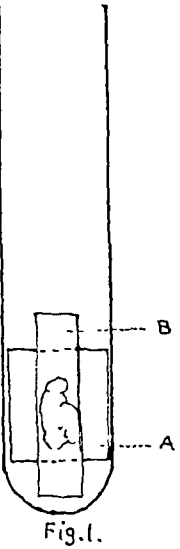


Fig. 1.

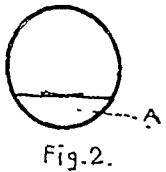


Fig. 2.

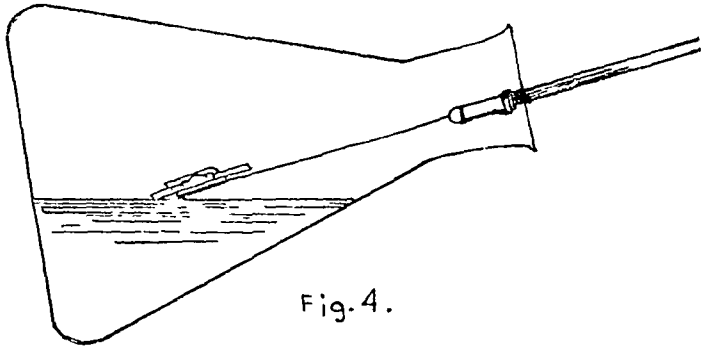


Fig. 4.

- Fig. 1. Tube containing vaseline-paper (A) and a strip of filter-paper (B) lying over it.  
 „ 2. Cross-section of a tube with the vaseline paper inside showing space (A) between its under-surface and the wall of the tube.  
 „ 3. The vaseline-paper and the filter-paper strip with culture lifted up with the aid of a platinum loop. The capillary attraction of the drop of liquid makes the paper stick to the loop and prevents it dropping off.  
 „ 4. The paper with the culture being laid on the surface of the broth.

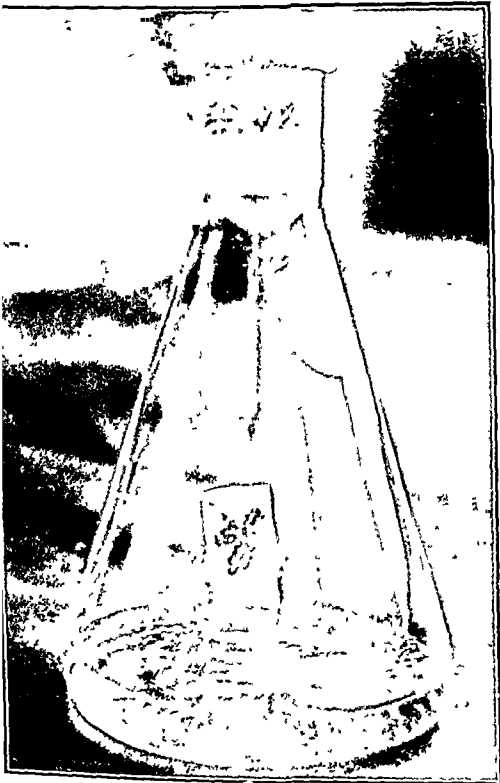


Fig. 5.

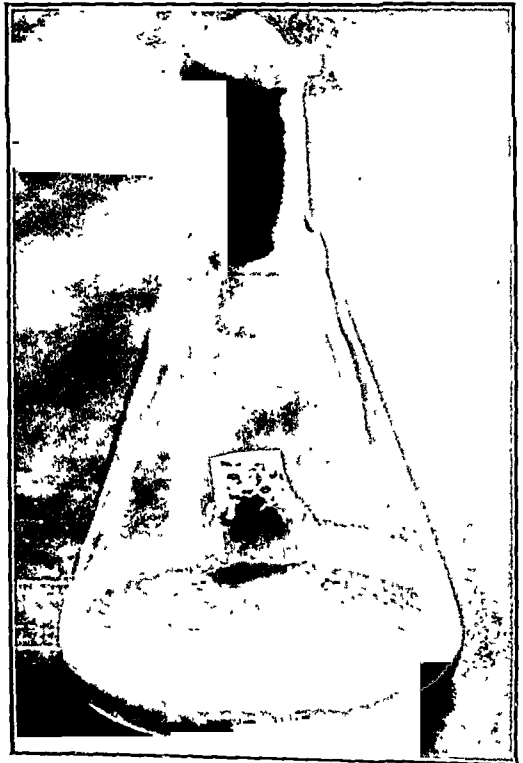


Fig. 6.

- Fig. 5. Conical broth flask showing a piece of filter-paper with the culture spread over it stuck to the inside of the wall of the flask with its lower end below the level of the broth. The growth is seen already commenced in the form of a thin surface film on the broth.  
 „ 6. A similar flask after three weeks more of incubation. The whole surface is seen covered with a thick wrinkled growth.

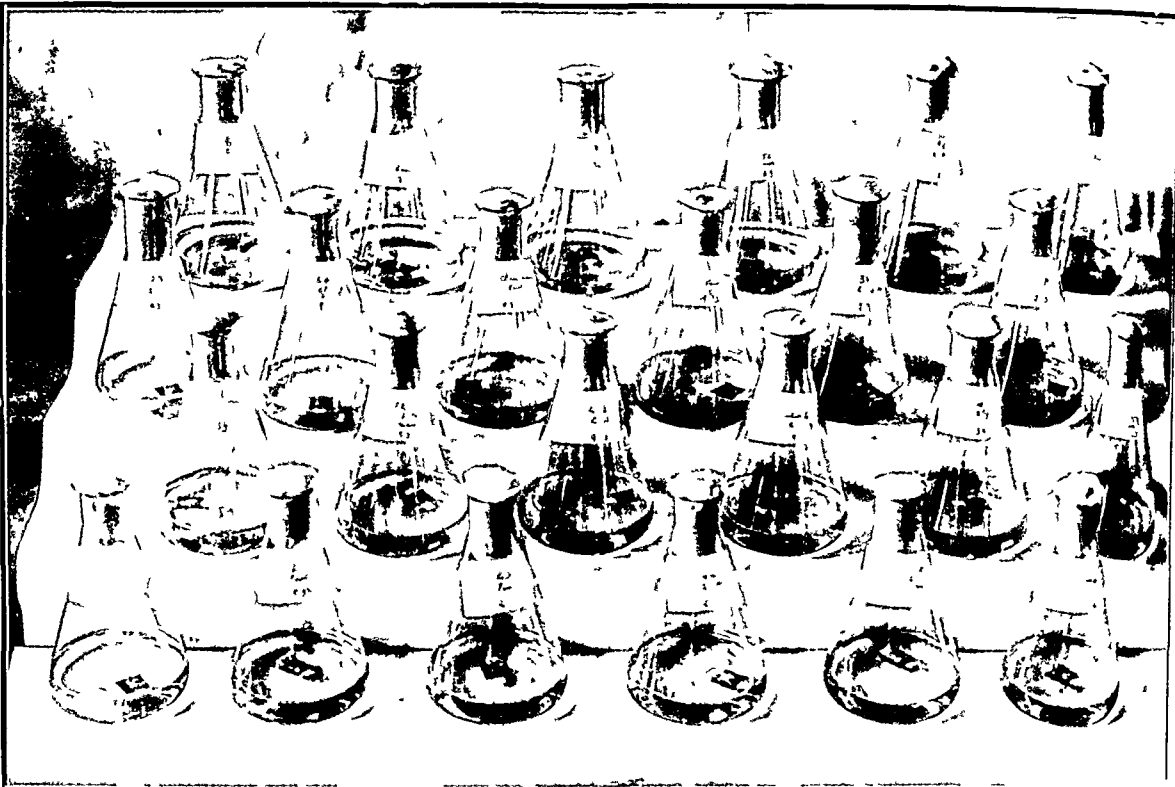


Fig. 7. Rows of flasks inoculated with different strains of tubercle bacilli. The culture on filter paper is seen floating on the broth (before incubation).

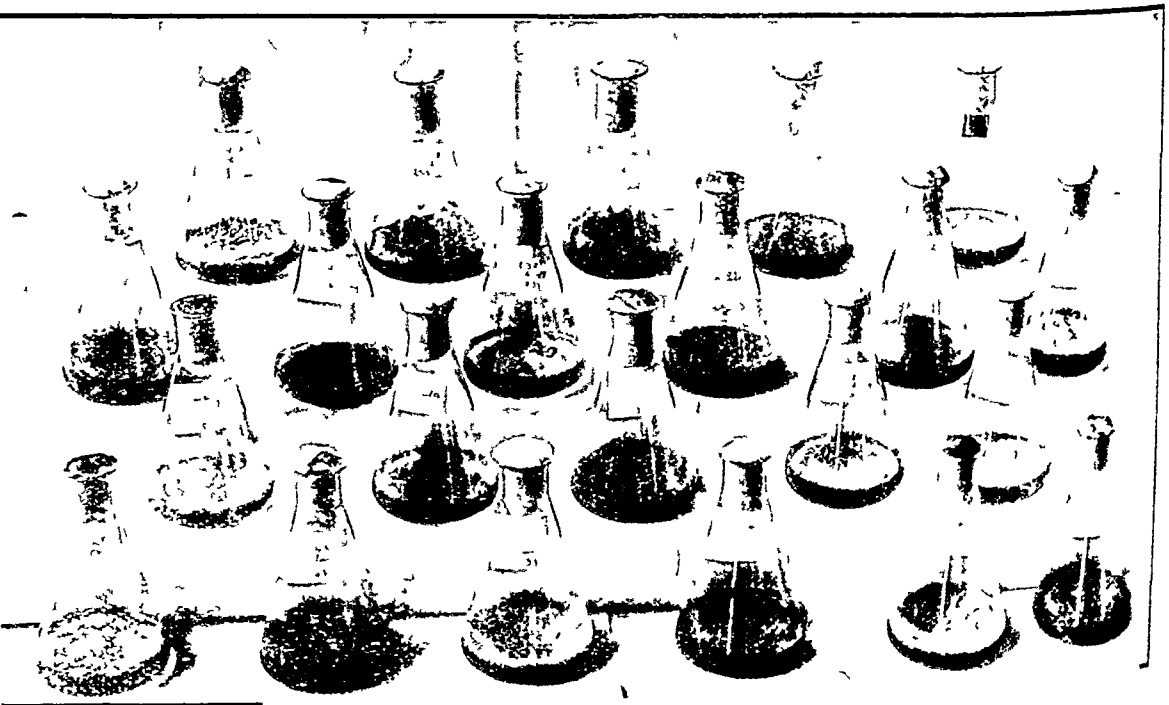


Fig. 8. Rows of flasks after four weeks' incubation, several of which are showing profuse surface growth.

broth by gently touching it with the paper and then lowering the end of the platinum loop beneath the surface of the broth, the flask being kept tilted nearly to a horizontal position for the purpose (Plate XXIV, fig. 4). If the platinum loop which is inserted underneath the vaseline-paper is charged with a drop of broth or salt solution, it will make the paper stick to the loop more firmly than otherwise and the chances of its dropping off the loop in the process of transfer are lessened (Plate XXIV, fig. 3). Several such flasks may thus be inoculated with different cultures and they are then gently removed without shaking and placed in the incubator.

The vaseline-paper continues to float on the surface and supports the filter-paper strip on which is deposited the seed culture and the extended ends of the latter allow, by their contact with the medium, its diffusion over the surface of the paper and promote growth which in course of time extends beyond the paper and ultimately covers the whole surface of broth.

If the filter-paper with the culture after being placed on the surface of the broth should happen to sink through some accident the culture can still be retrieved, provided, of course, it is still sticking to the filter-paper and is not washed off its surface, by pulling up with the aid of a long stout platinum wire loop the sunken filter-paper strip with the culture along the inner side of the flask and keeping it at such a level that the lower end of the strip remains in contact and about  $1/8$  to  $1/4$  inch below the level of the broth and the culture remains just above it. The result then resembles the second simpler method to be presently described. The other obvious remedy would naturally be to float another filter-paper strip with the culture in the same flask to replace the one that has sunken. More than one such strip may be floated in the same flask to give for each a separate island of growth. These islands will be scattered over the surface and will form several nuclei for fresh growth.

Stableforth (1929), describing the character of cultures of Johne's bacillus on broth, observes 'Surface cultures are difficult to start; usually some agent is necessary to enable the seed material to float and *yet be in contact with the medium*' (*italics* not in the original). This method fulfils these conditions. It has again several other features. It is simple and needs no elaborate preparations and can be carried out even in a small laboratory. The manipulations are easy and sterile conditions can be readily controlled.

Once the papers are kept ready, and they can be sterilized and stocked until needed, any number of cultures of growth of any description and consistency, whether scaly, moist or even slimy, can be readily transplanted directly from the solid to liquid media at any time without much preliminary preparation.

This method has been in use in our laboratory for some years and has proved very satisfactory.

Recently the writer has devised another and a still simpler method:—

In this method, the strip of filter-paper with the seed culture spread over its surface, instead of being floated over the broth, is stuck to the inner side of the conical flask containing the broth at such a level that the lower end of the paper strip remains in continuous contact and below the level of the broth. The wet strip with the culture thus remains stuck to the wall of the flask and the supply of



the medium to the seed through absorption by the filter-paper favours growth which gradually spreads over the surface of the liquid.

The details of the method are as follows :—

Ordinary filter-paper, about 0·15 mm. in thickness (a somewhat thicker paper is preferable), is cut into strips about three-fifth inch broad and about one-and-a-half inches long. The proper width of the strip is of importance for it should be only slightly smaller than the diameter of the tube in which it is kept to allow the platinum loop to be inserted with freedom under the paper for the purpose of its removal. If the strip is too narrow it will stick to the side of the tube when moistened and cause difficulty in its removal. These strips are distributed singly in test-tubes of about three-fourth inch diameter, sterilized in the autoclave and stored for use. When a broth culture is to be made one of these tubes is taken, the filter-paper is shaken and brought to near its mouth and is moistened with a drop of broth or salt solution but not so much as to make it stick to the sides of the tube. The seed culture from the solid medium is now placed in the centre and upper portion of the strip. By means of a thick platinum wire loop charged with a drop of salt solution and inserted under the paper the strip is then lifted up, removed from the tube and inserted in the flask to be inoculated and with a little manipulation made to stick to the inner side of the flask above the level of the fluid. The final position of the paper is then adjusted by means of the same thick platinum loop, so that a short length (about  $1/8$  to  $1/4$  inch) of its lower end remains in contact and below the level of the fluid ; the object being to maintain a continuous contact with a view to feeding the culture with the medium, the deposited culture being kept just above and touching the fluid. A rigid platinum wire (about 0·7 mm. to 0·8 mm. in thickness) is preferable for these manipulations as the one in common use easily bends. These manipulations are easily carried out in a conical flask but not so in a rounded or bulging flask for which the first method proves more convenient.

This method besides being simpler has a further advantage in that much less care is necessary than in the first method in handling and carrying the flasks to the incubator after inoculation to prevent the culture from sinking, as in this method the culture is no longer floating but is sticking to the side of the flask and if the flask is kept tilted in the opposite direction while being carried, so that the medium gravitates to the opposite side of the paper, the culture remains undisturbed.

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## THE NATURE OF IMMUNITY RESULTING FROM T. A. B. INOCULATION.

### A STUDY OF 'O' AGGLUTININS IN THE SERA OF INOCULATED HUMAN SUBJECTS AND THEIR IMMUNOLOGICAL SIGNIFICANCE.

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SINCE the Great War, prophylactic immunization in man against *B. typhosus*, *B. paratyphosus* A, and *B. paratyphosus* B has come to be practised almost universally. This procedure is dictated by the favourable results of statistical evaluation on the protective effects of T. A. B. inoculation as reflected in the rate of incidence and the case mortality rate of infections by these organisms (Leishman, 1921).

Immunological studies on the nature of immunity resulting from T. A. B. inoculation have given contradictory results. Felix (1924), Burnet (1924), and Stuart and Krikorian (1928) contended that the agglutinins produced in the sera of inoculated persons were almost entirely floccular in character—the 'H' agglutinins. These observations would annul the value of T. A. B. inoculation, since it has been demonstrated by immunity reactions, such as active immunization (Arkwright, 1927; Topley, 1929; Schütze, 1930), bactericidal action (Felix and Olitzki, 1926), and phagocytosis (Bhatnagar, 1935), that in the case of flagellated bacilli, as exemplified by the typhoid-paratyphoid group of organisms, the important factors from the point of view of immunity are the 'O' antigens and their corresponding antibodies. Their contentions have, however, been challenged, chiefly as they

relate to the diagnosis of enteric infections by the 'qualitative receptor analysis' method (Felix, *loc. cit.*) by various workers (Gardner, 1929; Whitehead, 1930; Smith, 1932; Mudd, 1932; and other workers). Lately, Giglioli (1933), Dulaney *et al.* (1933), and Dennis and Berberian (1934) have shown that in human subjects both 'O' and 'H' antibodies result from T. A. B. inoculation.

The present investigation was undertaken to study the 'O' agglutinins in the sera of inoculated persons and to assess their immunological significance. The 'H' agglutinins did not form part of the experiments to be described, since it has been shown, in the light of recently acquired knowledge of the antigenic structure of bacteria, that the flagellar 'H' antigens and the antibodies that react with them are of no protective value in active and passive immunization.

The sera were collected from a population which is inoculated every 18 months with two doses (0.5 c.c. and 1.0 c.c. at 10 days' interval) of a T. A. B. vaccine prepared by the Central Research Institute, Kasauli, to the following specifications:—

- (1) The organisms are grown on agar, heat killed (53°C. one hour) and carbonized (finished product adjusted to 0.5 per cent).
- (2) The strains employed are: (a) the classical Rawling's and (b) a recently isolated Indian 'Vi' strain. Both these are added in equal parts. (c) *B. paratyphosus* A (Mears) and (d) *B. paratyphosus* B (Rowlands). All these strains are rendered as 'smooth' as possible before use by broth passage, repeated plating and selection of colonies.
- (3) The vaccine as issued contains per c.c.:—

<i>B. typhosus</i> .. ..	1,000 millions.
<i>B. paratyphosus</i> A ..	750 "
<i>B. paratyphosus</i> B ..	750 "

## TECHNIQUE.

### 1. Agglutination tests.

Living bacteria grown 18 to 24 hours on agar slopes, prepared from fresh meat bouillon, and suspended in fresh normal saline, were employed for these tests. One drop of such a suspension, twenty times as thick as that used for this test according to Dreyer's technique, was added to the serum dilutions contained in a total volume of 1 c.c. in tubes with a uniform internal diameter. The tubes were incubated for two hours at 37°C. and left for a further 22 hours at room temperature. The final reading was then taken.

The strains employed for these tests were Ty 901 (O901 and H901) (Weil and Felix, 1920), Watson (Perry, Findlay and Bensted, 1933a), *Paratyphosus* A (OAK) obtained from the Enteric Laboratory, Kasauli, and *B. paratyphosus* B—OB—(Schütze, *loc. cit.*).

All the strains were grown at 37°C., sub-cultured daily and kept under permanent control for smoothness by plating once a week and picking out perfectly smooth colonies for further sub-culturing. In addition to normal saline control



It will be noted that the 'Vi' antibody was detected to a titre of 1 : 50 in the sera of two out of fifty individuals. Both of them gave a history of having suffered from a 'prolonged fever' six months and ten months ago respectively. As Felix, Krikorian and Reitler (1935) have recently drawn attention to the 'possible relationship between 'Vi' agglutinins and the diagnosis of carrier condition, these two individuals were tested carefully for the presence of the enteric group of organisms in their stools and urine. The results were, however, negative.

It was noticed that the figures reported in Table I, what may be termed the normal 'O' agglutinins in a random sample of population, were very much higher than those recorded by observers in other countries and in India (Gardner and Stubington, 1932; Amzel *et al.*, 1934; Lewin, 1934; Bole, 1935; Pasricha *et al.*, 1936; and other workers). An explanation of this discrepancy was, therefore, sought.

Two differences were evident in the technique for agglutination, namely, (1) while live organisms were used by us, killed and preserved emulsions were employed by most other workers, and (2) our tests were set up in tubes of wide internal diameter (1.1 c.c.) with a round bottom. So far as can be judged by the study of published reports, Dreyer's tubes were used by others.

A comparative series of tests were, therefore, carried out on 44 normal human sera to elucidate whether there was any difference due to these two factors.\* The results obtained with 10 sera are incorporated in Table II. The other sera gave figures of a similar order. They are not included here on account of economy of space.

TABLE II.

*The agglutinability of B. typhosus by normal human sera.*

Normal human sera.	AGGLUTINATION OF STRAIN 0901 OF <i>B. typhosus</i> .		
	Live.	KILLED AND PRESERVED	
		in tubes as described in the text.	in Dreyer's tubes.
1	250	150	30
2	200	150	50
3	200	100	25
4	125	75	15
5	100	50	Nil
6	75	45	15
7	75	50	Nil
8	50	30	Nil
9	50	25	Nil
10	25	Nil	Nil

\* These tests were carried out by the senior author in collaboration with Lieut.-Colonel R. F. Bridges, R.A.M.C., Officer-in-charge, Enteric Laboratory, Kasauli, by kind permission of the Director of Medical Services in India.

scrupulous cross-examination, any previous history of T. A. B. inoculation was excluded. The results are recorded in Table I:—

TABLE I.

'O' agglutinins for *B. typhosus*, *B. paratyphosus A*  
and *B. paratyphosus B* in the sera of  
50 uninoculated individuals.

Type of antibody.	Titre of antibody.	Number of individuals.
T. O. {	25	5
	50	12
	100	32
	200	1
A. O. {	25	37
	50	12
	100	1
	200	..
B. O. {	25	12
	50	36
	100	2
	200	..
'Vi' {	10	..
	25	..
	50	2
	100	..

*Note.*—The figures represent the denominators of titres, i.e., highest dilution of serum in which a partial (+) agglutination was observed by the naked eye according to the following scheme:—

- +++ Plentiful sediment, supernatant fluid clear.
- ++ Plentiful sediment, supernatant fluid turbid.
- + Small sediment, flakes in supernatant fluid.

Observed with 10 × lens {  $\pm$  Very small flakes in supernatant fluid.  
                           $\pm$  Trace.  
                           $\pm$  Faint trace.

It is seen that all the sera examined possessed the 'O' antibody, for the three types of organisms under consideration, to a titre varying from 1:25 to 1:200. The agglutinins for *B. typhosus* and *B. paratyphosus B* were found to be present in a much higher proportion than those for *B. paratyphosus A*. The variation in the sensitivity of the organisms employed for agglutination may account for these differences to a certain extent. The comparative low agglutinogenic capacity of *B. paratyphosus A*, demonstrated in another communication (Bhatnagar, Freeman and Gera, 1937), and its bearing on the presence of normal agglutinins for this organism will be discussed later.

TABLE III—concl'd.

Type of anti-body.	Titre of anti-body.	INTERVAL AFTER T. A. B. INOCULATION.			
		3 months.	6 months.	12 months.	18 months.
A. O.	25	—	1	4	5
	50	—	1	—	11
	100	3	9	17	8
	200	9	7	4	1
	300	10	7	—	—
	500	3	..	—	—
	750	—	..	—	—
B. O.	25	1	1	6	7
	50	—	1	2	10
	100	2	5	7	7
	200	4	4	7	1
	300	5	6	3	—
	500	9	7	—	—
	750	—	1	—	—
'Vi'	1,000	4	—	—	—
	10	—	Total number of sera examined for 'Vi' antibody = 100.		
	25	4			
	50	1			
	100	—			

It will be seen that 'O' agglutinins for all the three members of the typhoid-paratyphoid group of organisms were found to be present in the sera of all the individuals tested, the titre varying from 1 : 25 to 1 : 1,500. As noted already amongst the uninoculated, so also here in the case of inoculated persons, *B. typhosus* and *B. paratyphosus* B registered higher content of 'O' agglutinins as compared with *B. paratyphosus* A.

A definite time relationship between the titres of all the three types of agglutinins and the date of inoculation are evident from a perusal of Table III. The titres fall as more and more time elapses after inoculation, so that at the end of 12 months they, more or less, approximate to those which have been found to exist amongst the uninoculated individuals (*vide* Table I).

Out of 100 sera examined in this series, the 'Vi' antibody was found to be present in 5 cases to a titre of 1 : 25 (4 cases) and 1 : 50 (one case). On inquiry, all these individuals gave a history of having suffered from a 'prolonged fever' during the last two years.

#### THE DEVELOPMENT OF 'O' AGGLUTININS IN RESPONSE TO T. A. B. INOCULATION.

The sera of 50 persons, who were due for T. A. B. inoculation, i.e., 18 months after the last course, were collected just before the first dose of the new course and

It will be seen that the live organism proved to be more sensitive than its killed and preserved emulsion. The difference, however, was not such as to justify the high readings reproduced in Table I.

On the other hand, when 'O' agglutination was carried out in Dreyer's tubes and those used by us, the difference between the two readings was very significant. In the latter type of tube, the unagglutinated organisms deposit as a dense circular mass in the centre of the round bottom, while the agglutinated bacteria come down as small granular particles which settle all round the lowest part of the tube. It is thus easy to differentiate between the agglutinated and the non-agglutinated sediment either by the naked eye or with the help of a magnifying lens (10 ×) depending upon the amount of agglutination. Confirmation of this fact is obtained by shaking the tube when the unagglutinated organisms disperse as a homogeneous opacity while the agglutinated granules float in the opaque suspension as such.

Compared with this method, agglutination carried out in Dreyer's tubes suffers from the defect in that it is impossible to say how much of the sediment consists of agglutinated organisms and how much is a pure deposit of non-agglutinated bacteria.

Differences of a similar nature were noticed when normal human sera were titrated for their 'O' agglutinin content against *B. paratyphosus* A and *B. paratyphosus* B. That agglutination in these tubes was not of a non-specific nature was eliminated by absorption tests. It must, however, be stated that when high titre immunized rabbit sera were tested side by side in the two types of tubes, the difference in the titres obtained was much less.

#### THE PRESENCE OF 'O' AGGLUTININS IN THE SERA OF INOCULATED HUMAN SUBJECTS.

Twenty-five sera of each were collected at random from persons who had been inoculated 3 months, 6 months, 12 months, and 18 months previously and titrated for the presence of 'O' agglutinins. The results are reproduced in Table III:—

TABLE III.

'O' agglutinins at different intervals after T. A. B. inoculation.

Type of anti-body.	Titre of anti-body.	INTERVAL AFTER T. A. B. INOCULATION.			
		3 months.	6 months.	12 months.	18 months.
T. O.	25	—	—	—	—
	50	—	—	1	4
	100	1	—	5	10
	200	1	4	11	9
	300	—	—	8	2
	500	10	18	—	—
	750	8	2	—	—
	1,000	4	1	—	—
	1,500	1	—	—	—



TABLE IV—*concl'd.*

Number.	TITRE OF ANTIBODIES BEFORE T. A. B. INOCULATION.				TITRE OF ANTIBODIES AFTER T. A. B. INOCULATION (25 DAYS AFTER SECOND DOSE).			
	T. O.	A. O.	B. O.	'Vi'	T. O.	A. O.	B. O.	'Vi'
25	200	100	100	0	750	200	1,000	0
26	100	50	50	0	500	300	500	0
27	200	100	100	0	200	200	200	0
28	100	50	100	0	500	500	1,000	0
29	300	100	50	0	1,500	300	50	0
30	100	50	100	0	1,000	200	1,000	0
31	100	25	50	0	200	100	200	0
32	200	200	100	0	200	200	100	0
33	50	100	100	0	1,000	200	500	0
34	50	50	100	0	500	300	1,000	0
35	100	100	50	0	500	200	500	0
36	200	25	50	0	1,000	100	500	0
37	100	100	50	0	500	300	1,000	0
38	100	25	50	0	1,000	200	750	0
39	50	50	100	0	500	300	500	0
40	200	50	50	0	1,500	200	500	0
41	200	25	50	0	500	200	500	0
42	200	100	100	0	750	300	750	0
43	300	25	100	0	500	300	1,000	0
44	100	50	50	0	1,000	100	300	0
45	50	50	50	0	500	200	500	0
46	100	100	100	0	1,000	300	500	0
47	100	200	100	0	500	200	1,000	0
48	300	50	50	0	1,000	200	500	0
49	200	100	100	0	750	300	1,000	0
50	100	100	50	..	1,500	200	500	0

Note.—Titre '0' equals a negative result in dilution 1 : 10.

It was considered that the development of 'O' agglutinins will be demonstrated better if the titres of this antibody, before and after T. A. B. inoculation, were shown, side by side, in all the sera examined. This procedure, however, resulted in a lengthy table. An analysis of the figures shown therein was, therefore, made and is reproduced in Table V :—

TABLE V.

*'O' and 'Vi' agglutinins before and after T. A. B. inoculation.*

Type of antibody.	BEFORE T. A. B. INOCULATION.		AFTER T. A. B. INOCULATION.	
	Titre.	Number of individuals showing the particular titre. (Total 50).	Titre.	Number of individuals showing the particular titre. (Total 50).
T. O.	50	10	200	5
	100	19	500	20
	200	16	750	7
			1,000	12
	300	5	1,500	6
A. O.	25	8	100	7
	50	17	200	25
	100	19	300	14
	200	6	500	4
B. O.	25	2	100	2
	50	21	200	7
	100	22	300	4
	200	5	500	23
	..	..	750	2
	..	..	1,000	12
'Vi'	10	—	10	—
	25	—	25	—
	50	—	50	—
	100	—	100	—

It was next decided to test the production of 'O' antibody in response to primary T. A. B. inoculation. Twenty-five sera were examined in this connection. The results are included in Table VI. It will be seen that while a considerable amount of 'O' antibody is elaborated for all the three types of organisms, the height of the titres attained, however, falls significantly short of that obtained when the same procedure is adopted in the case of those who have been inoculated before (*cf.* Tables V and VI).

To test that these differences were not due to the particular type of vaccine employed, 10 sera were collected from individuals who had been inoculated six

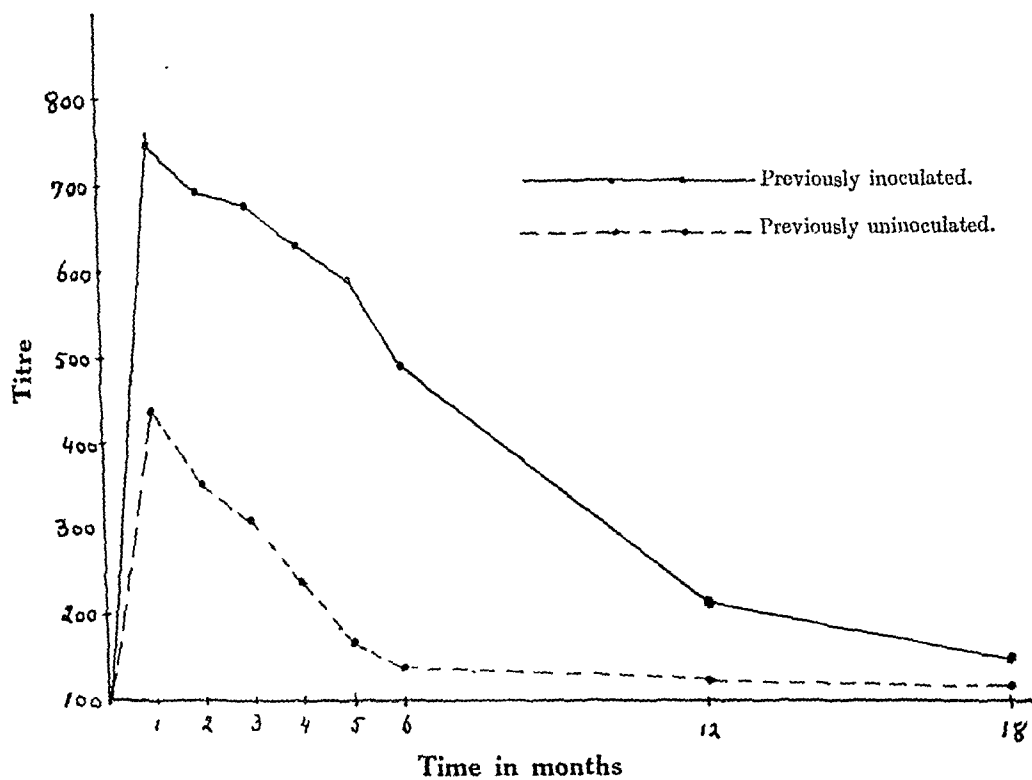


Fig. 1. The production of 'O' agglutinins in human subjects: showing difference in response to primary and subsequent T. A. B. inoculation.

months previously in England with a T. A. B. vaccine prepared by the Royal Army Medical College, Millbank, London, and referred to in various communications by Perry, Findlay and Bensted (1933*a*, *b*; 1934*a*, *b*). Identical results were obtained, the agglutinins being as low six months after primary inoculation with Millbank vaccine as with Kasauli vaccine.

The fall of T. O. agglutinins was next studied in the two types of individuals (primary inoculation versus subsequent inoculation) by bleeding them every month up to six months. The results are charted in Fig. 1 in terms of average titres

TABLE VI.

'O' agglutinins after primary T. A. B. inoculation. Inoculated 6 months previously, 20. Inoculated 12 months previously, 15. Inoculated 18 months previously, 15. Total number examined = 50.

Type of antibody.	Titre of antibody.	INTERVAL AFTER PRIMARY T. A. B. INOCULATION.			Twenty-five sera examined 25 days after primary T. A. B. inoculation.
		6 months.	12 months.	18 months.	
T. O. {	50	2	1	2	—
	100	10	12	11	—
	200	7	2	2	3
	300	1	—	—	5
	500	—	—	—	16
	750	—	—	—	1
	1,000	—	—	—	—
A. O. {	25	2	4	2	—
	50	8	8	12	2
	100	9	3	1	4
	200	1	—	—	15
	300	—	—	—	4
	500	—	—	—	—
B. O. {	25	—	—	—	—
	50	5	6	5	—
	100	6	7	8	2
	200	8	2	2	8
	300	1	—	—	9
	500	—	—	—	6
	750	—	—	—	—

When this table is compared with Table III, which records similar intervals in the case of those who have had more than one course of T. A. B. inoculation, a definite difference is evident. It is seen that, after primary inoculation, the titre for all the three types of agglutinins has, by the end of six months, fallen to a level which, more or less, corresponds to that found amongst uninoculated persons (*vide* Table I). Such, however, is not the case with those who have had multiple inoculations, as seen from a comparison of the average titres:—

Average titre six months after inoculation.			Primary inoculation.	Subsequent inoculation.
T. O.	..	..	140	410
A. O.	..	..	78	260
B. O.	..	..	138	460

experiments on laboratory animals, that the injection of a large dose of antigen may be followed by a well-marked negative phase, advantage was taken to test if this was the explanation of the above coincidence. At the same time the effect of two doses of T. A. B. vaccine—0.5 c.c. and 1.0 c.c.—on the elaboration of 'O' agglutinins was determined separately, since it occurred to us from the data collected by Ledingham and Schütze (1931) that after secondary and subsequent responses the antibody-forming mechanism may get so sensitized that a small stimulus, like the first dose of T. A. B. vaccine, may lead to the production of a large enough amount of 'O' antibody thus making the second injection unnecessary.

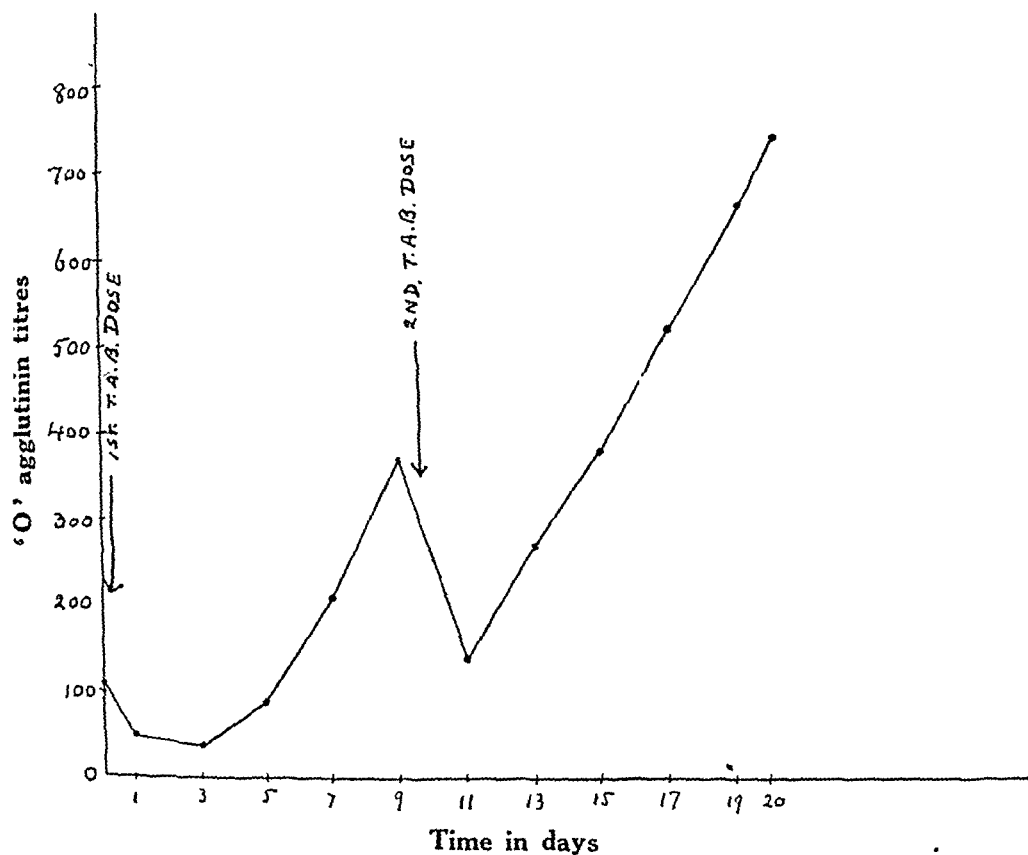


Fig. 2. Agglutinin production: response of previously inoculated persons to T. A. B. inoculation.

Individuals for this experiment were, therefore, selected from those who had had more than five courses of T. A. B. inoculation. They were bled before the commencement of the new course and every second day till ten days after the second dose. Their average titres for T. O. agglutinins are charted in Fig. 2.

It is noticed that each injection results in a temporary decrease in the titre of 'O' antibody. The induction or the negative phase lasts about four to five days

obtained from a number of individuals which in no case was less than 25. At the same time, the average titres at the end of 12 and 18 months are included for comparison. Both the titres and the time are plotted on an arithmetical scale in preference to a logarithmic scale as with the latter small differences at the bottom of the scale are disproportionately enhanced in graphic representation, whilst large differences at the summit of the curve are minimized.

It is seen from this figure that, in a person who has been inoculated before, the curve of 'O' agglutinin titre tends not only to rise higher than in one who has not been inoculated before, but also to remain high over a longer period.

The fall of agglutinins in the two types of individuals is compared in Table VII where the residual agglutinins are represented in percentages of the highest average titre resulting from T. A. B. inoculation:—

TABLE VII.

*The fall of agglutinins (a) after primary inoculation  
and (b) after subsequent inoculation.*

Period after T. A. B. inoculation in months.	RESIDUAL AGGLUTININS AFTER T. A. B. INOCULATION IN INDIVIDUALS.	
	Inoculated primarily, per cent.	Inoculated previously, per cent.
3	71	90
6	32	66
12	27	28
18	24	20

It will be seen that while the titres correspond at the end of 12 months, the maximum fall in the case of those having their first inoculation takes place within the first six months. In the previously inoculated individual, on the other hand, the fall is gradual and a fairly high titre is maintained for about 12 months. From the end of the year onwards, the titre in both types of individuals runs, more or less, parallel on a level which corresponds with that found in persons who have never been inoculated.

*The 'negative phase' and the effect of two doses on the production of  
'O' agglutinins.*

Cases of enteric group of fevers have lately come to notice where the natural infection followed soon after the T. A. B. inoculation in as high a proportion as 10 per cent of the total number inoculated. In view of what is known from

experiments on laboratory animals, that the injection of a large dose of antigen may be followed by a well-marked negative phase, advantage was taken to test if this was the explanation of the above coincidence. At the same time the effect of two doses of T. A. B. vaccine—0.5 c.c. and 1.0 c.c.—on the elaboration of 'O' agglutinins was determined separately, since it occurred to us from the data collected by Ledingham and Schütze (1931) that after secondary and subsequent responses the antibody-forming mechanism may get so sensitized that a small stimulus, like the first dose of T. A. B. vaccine, may lead to the production of a large enough amount of 'O' antibody thus making the second injection unnecessary.

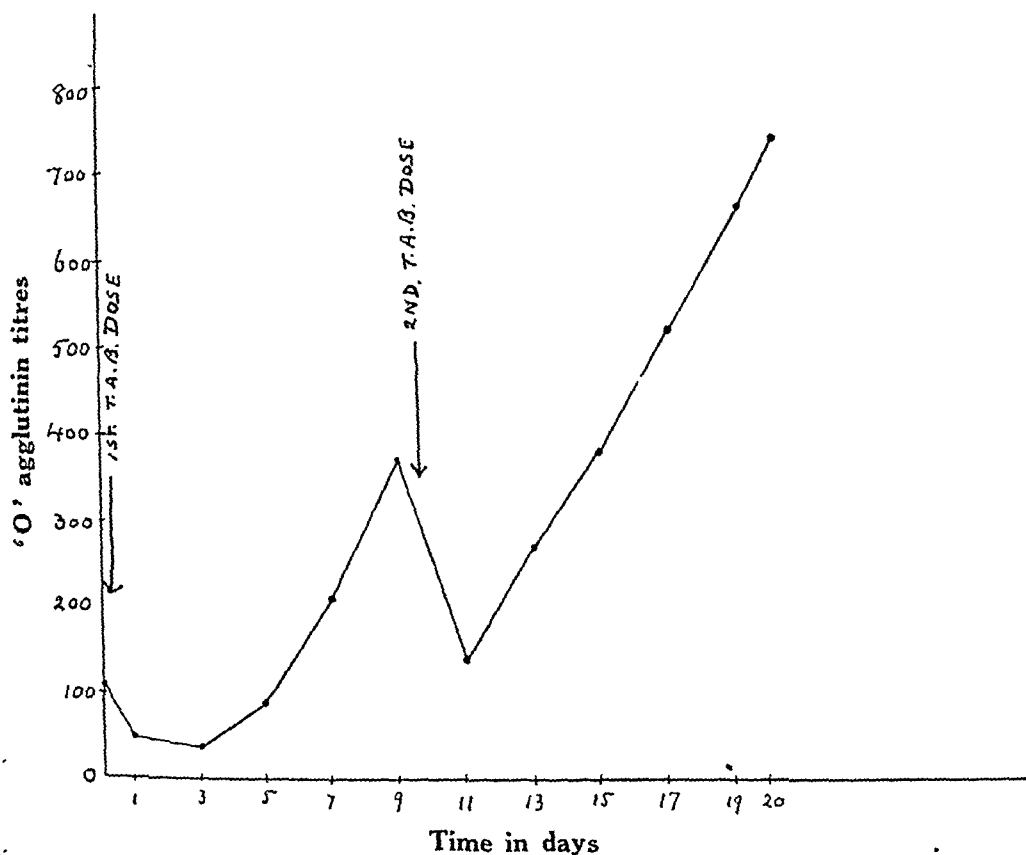


Fig. 2. Agglutinin production: response of previously inoculated persons to T. A. B. inoculation.

Individuals for this experiment were, therefore, selected from those who had had more than five courses of T. A. B. inoculation. They were bled before the commencement of the new course and every second day till ten days after the second dose. Their average titres for T. O. agglutinins are charted in Fig. 2.

It is noticed that each injection results in a temporary decrease in the titre of 'O' antibody. The induction or the negative phase lasts about four to five days

both with the first as well as with the second dose, this being the time taken by the titre to reach the same level as that before the inoculation. In no case was a prolonged negative phase noticed, although, as stated before, in 10 to 15 per cent of individuals examined the highest level of 'O' titre did not rise higher than what is met with amongst uninoculated individuals. A summation of the agglutinin producing effect by two doses is seen from Fig. 2 to take place, the second dose raising the titre to a height which is more than double of what resulted from the first dose. It is thus shown that repeated T. A. B. inoculations do not lead to a change in the reactivity of the antibody-forming apparatus such as results when infection takes place with a living virus; nor does a negative phase play any rôle in this type of immunization.

*The immunological significance of 'O' agglutinins.*

Having studied the manner in which 'O' agglutinins are elaborated in response to T. A. B. inoculation, it was next considered desirable to determine if they afforded any protection in terms of two well-known immunity reactions, namely, phagocytosis and passive immunization. For comparison, sera from a rabbit immunized with identical doses of T. A. B. vaccine and from a typhoid patient were included. Although the 'O' antibody content of the three sera differed it was so adjusted by dilution that the final concentration of this antibody was the same in all the sera tested. The presence of 'Vi' antibody was excluded in as low a dilution as 1 : 5, whilst the bearing of 'H' agglutinins on protection tests, such as is attributed by Springut (1927) to this antibody, was annulled by the fact that its titre, more or less, corresponded in the three types of sera.

A strain of intermediate type (Felix, Bhatnagar and Pitt, *loc. cit.*) of *B. typhosus* (Ty Razmak A) was utilized for these tests. The results of experiments are summarized in Table VIII:—

TABLE VIII.

*Phagocytosis and passive immunization with 'O' antibody of animal and human origin.*

Sera.	Titre of 'O' agglutinins.	PHAGOCYTOSIS OF STRAIN TY RAZMAK A.		PASSIVE IMMUNIZATION OF MICE.	
		Number of organisms phagocyted by 100 leucocytes.	Cells taking part, per cent.	Dose of serum in c.c.	Protected against
T. A. B. rabbit ..	500	110	56	0.25	4 M. L. D.
T. A. B. human ..	500	148	70	0.25	6 M. L. D.
Typhoid patient .	500	230	98	0.25	10 M. L. D.

*Note.*— 1 M. L. D. =  $200 \times 10^6$  organisms of strain Ty Razmak A.



This table demonstrates that all the sera with an 'O' antibody content of 500 agglutinins opsonize and protect to a significant degree. A striking discrepancy, well marked in both the immunity reactions, is, however, shown by the three types of sera. The 'O' antibody resulting from a natural infection (typhoid patient's serum) is seen to be far superior both in its phagocytosis promoting action as well as in passive protection, when compared to the same antibody elaborated in response to T. A. B. inoculation. Even with the latter a difference is evident, the 'O' antibody of human origin recording better results than one produced in a rabbit with identical procedure.

These results indicate that inherent qualitative differences exist in the 'O' antibody produced by various immunizing processes. The immunity conferred by a living bacterial cell (typhoid patient's serum), measured in terms of 'O' antibody, cannot be approached by artificial immunization with a killed antigen, although the agglutinin titres may be identical in the two cases. Similarly, the slight superiority of 'O' antibody of T. A. B. human serum may be due to the fact that this antibody is not a pure product of immunization since it contains a large component which comes from natural sources. It is contended that these experiments offer an explanation of the lack of parallelism between antibody titre and the power of protection, observed by many workers, where the source of origin of the immune body was not taken into consideration.

#### DISCUSSION.

The results of the experiments on the sera of over 500 persons, presented in this paper, have a direct bearing on the theory and practice of T. A. B. inoculation. In conformity with other observers (Giglioli, *loc. cit.*; Dulaney *et al.*, *loc. cit.*; Dennis and Berberian, *loc. cit.*) it has been shown that inoculation with T. A. B. vaccine results in the production of a considerable amount of 'O' antibody, the immunological significance of which, and that of the smooth 'O' antigen that gives rise to it, has been well established in prophylaxis by Felix (*loc. cit.*), Felix and Olitzki (*loc. cit.*), Arkwright (*loc. cit.*), Ibrahim and Schütze (1928), Topley (*loc. cit.*), and others.

It is observed that 'O' agglutinin production by *B. paratyphosus* A falls significantly short as compared with the other two organisms included in a T. A. B. vaccine. A similar statement is recorded by Perry and Bensted (1929). From a study of its agglutinogenic activity in laboratory animals, Bhatnagar, Freeman and Gera (*loc. cit.*) concluded that a low antigenic response was the inherent property of the smooth 'O' antigen of this organism since it was noticed when immunization was carried out with live, heated and phenolized suspensions administered by either the intravenous or subcutaneous route.

A fundamental difference between primary and secondary response to T. A. B. inoculation in human subjects has been demonstrated. With a primary stimulus, the maximum rise of 'O' antibody is much less than that obtained with secondary and subsequent stimuli. Besides, the fall of titre is much quicker in the former case, so much so, that at the end of six months the 'O' antibody titre corresponds to that met with amongst uninoculated individuals (*vide* Tables I and VI, and Fig. 1). This difference finds its explanation in the fact that the first T. A. B. inoculation

confers an increased power of response to any identical subsequent stimulation. As it is generally accepted that the maintenance of immunity in the general population is due in some degree to repeated antigenic stimuli given by sub-clinical doses of natural infection, this observation is all the more interesting, since the individuals experimented on had ample opportunities of being exposed to enteric infection in their early life, so that the primary stimulation of the antibody-forming apparatus in this direction did not commence with the first administration of T. A. B. vaccine.

Support for our view is forthcoming (a) from similar experience in laboratory animals (Glenny and Sudmersen, 1921 ; Glenny, 1925 ; Topley, 1933) and (b) from a statistical analysis of epidemic data of enteric group of fevers amongst British troops in India (Report on the Health of the Army, War Office, London, 1933). Experience in the army in India shows definitely that there is a higher incidence of enteric during the first year of service of British troops in the country than in later years. This fact may be in a considerable degree due to the practice of repeating T. A. B. inoculation annually with the result that an added amount of immunity is obtained later. There is of course the possibility, mentioned above, that in Indian surroundings the antigenic stimulus suggested may play a part, this representing what might almost be called 'acclimatization'.

Before 1929 the population under consideration was given a single 1 c.c dose of T. A. B. vaccine every 12 months. Since then, two doses—0.5 c.c. and 1.0 c.c.—are administered every 18 months. Our observations on 'O' agglutinin titres at different periods after inoculation, primary or secondary, very strongly suggest that the interval between a first inoculation and re-inoculation is longer than is advisable.

The inclusion of an effective 'Vi' fraction of *B. typhosus* in the T. A. B. vaccine would be a step in the right direction so that a vaccine of maximum immunizing value might be produced. It was first shown by Felix and Pitt (1934), by experiments on mice, that this new antigen shares its place with the smooth 'O' antigen in virulence and in protective action. These observations have since been confirmed by the study of pathogenesis of typhoid fever in man (Kaufmann, 1935; Felix, Krikorian and Reitler, *loc. cit.*). Our experiments, however, indicate that when a heat-killed and carbolized vaccine prepared from a 'Vi' strain is employed, the human body fails to elaborate the 'Vi' antibody even to as low a titre as 1:10 (Table IV). The explanation of this anomaly is to be found in the harmful effect on the 'Vi' antigen of exposure to the action of phenol pointed out by Felix and Bhatnagar (*loc. cit.*). Stuart and Krikorian (*loc. cit.*) have similarly drawn attention to some deleterious effect of phenol on the 'O' antigen of *B. typhosus*. Taken in conjunction with these two statements, the facts recorded in this communication present a strong case for renewed efforts in search of a preservative which will leave the immunogenic value of the antigenic components of T. A. B. vaccine unimpaired.

Apart from the immediate bearing these observations have on the special problem of T. A. B. inoculation, they are of a more general immunological interest as well. Felix and Bhatnagar (*loc. cit.*) referred to a phenomenon of 'functional deficiency' in the case of the 'Vi' antibody. They established that this antibody resulting from immunization with formalized extracts of virulent cultures of

*B. typhosus* showed a well-marked discrepancy between its agglutinin titre and its protective power, when compared to the same antibody formed in response to immunization with the live organisms. The experiments summarized in Table VIII show a striking difference of a similar nature in the behaviour of 'O' antibody produced against a killed antigen and that developed in response to a natural infection. As many weighty inferences are drawn in immunological investigations from the presence of agglutinins, it is suggested that the lack of relationship between the antibody titre and the power of protection, so far as it relates to those antibodies which are of value in immunity, noted by many observers, may find an important explanation in the physical and chemical processes to which an antigen is subjected before being utilized as an immunizing agent.

#### SUMMARY.

(1) T. A. B. inoculation leads to the production of a considerable amount of 'O' antibody. In the case of *B. paratyphosus* A this response is not evident to the same extent as that for the other two organisms.

(2) A fundamental difference exists between primary and secondary response to T. A. B. inoculation in human beings. With a primary stimulus the maximum rise of 'O' antibody titre falls significantly short of that obtained with secondary and subsequent stimuli. It is, therefore, suggested that the second T. A. B. inoculation should be practised at the end of six months.

(3) In those individuals who have had multiple inoculations, the 'O' agglutinin titre remains fairly high for 12 months only. The desirability of repeating T. A. B. inoculation every year is, therefore, stressed.

(4) In spite of the inclusion of a 'Vi' strain of *B. typhosus*, the present T. A. B. vaccine does not produce any 'Vi' antibody. The explanation of this anomaly is given.

(5) Qualitative differences in 'O' antibody resulting from various immunizing processes are demonstrated. An explanation of the lack of relationship between the titre of this antibody and its behaviour in immunity reactions is advanced.

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## THE AGGLUTINOGENIC AND AGGLUTINATING ACTIVITIES OF *B. PARATYPHOSUS* A.

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IN another communication (Bhatnagar, Freeman and Dhilon, 1937), while studying the nature of immunity resulting from T. A. B. inoculation in human beings, it was found that the production of 'O' agglutinins against *B. paratyphosus* A was significantly lower as compared with those for the other two organisms. On account of the practical importance of this observation, the agglutinogenicity and the agglutinability of this member of the typhoid-paratyphoid group were made the subject of a short study.

Three strains of *B. paratyphosus* A were selected for this purpose, viz., (i) *Mears* which is incorporated by many laboratories in various parts of the world in their T. A. B. vaccine, and (ii) and (iii) two other strains (*ASA* and *RK*) recently isolated by this laboratory from human cases.

Rabbits were immunized with these strains intravenously, the suspensions used being (a) live, (b) heated at 53°C. for one hour, (c) heated at 53°C. for one hour and then carbolized (0.5 per cent), and (d) steamed for one hour at 93°C., this being the temperature at which water boils in Razmak (height 6,556 feet above sea-level). The total dosage in each case was  $1,200 \times 10^6$  organisms divided into three doses of 200, 400, and 600 million organisms administered at two-day intervals. The animals were bled on the sixth day after the last injection.

The immune sera thus obtained were tested against a strain of *B. paratyphosus* A (*OAK*) particularly sensitive to 'O' agglutination and supplied to us by the courtesy of Lieut.-Colonel R. F. Bridges, R.A.M.C., Officer-in-charge, Enteric Laboratory, Kasauli. The technique of agglutination followed was that described by Felix and his co-workers.

In the experiments to be described, 'H' agglutination was not estimated as it is recognized that the flagellar 'H' antigens and the antibodies that react with them play no important part in infection and in immunity.

THE PRODUCTION OF 'O' AGGLUTININ ON IMMUNIZATION WITH  
DIFFERENT VACCINES OF *B. paratyphosus A.*

TABLE I.

*A comparison of 'O' antibody production by B. paratyphosus A,  
B. typhosus and B. paratyphosus B.*

Number of rabbit.	IMMUNIZATION OF RABBITS WITH VARIOUS STRAINS.			TITRE OF 'O' ANTIBODY.	
	Type of organism.	Strain.	Dose in millions of organisms.	Injected intra-venously.	Injected subcutaneously.
41	<i>B. paratyphosus A.</i>	Live .. { <i>Mears</i>	1,200	3,000	1,000
46			<i>ASA</i> 1,200	5,000	..
47			<i>RK</i> 1,200	5,000	..
42		Heated at 53°C. for one hour. { <i>Mears</i>	1,200	1,000	500
49			<i>ASA</i> 1,200	2,000	..
51			<i>RK</i> 1,200	3,000	..
43		Phenolized .. { <i>Mears</i>	1,200	1,000	500
50			<i>ASA</i> 1,200	2,000	..
53			<i>RK</i> 1,200	3,000	..
44		Heated at 93°C. for one hour. { <i>Mears</i>	1,200	100	50
54			<i>ASA</i> 1,200	150	..
52			<i>RK</i> 1,200	200	..
57	<i>B. typhosus</i>	Ty Razmak, live ..	1,200	20,000	10,000
58		Ty Razmak heated at 53°C. for one hour.	1,200	10,000	7,500
59		Ty Razmak phenolized	1,200	10,000	10,000
60		Ty Razmak heated at 93°C. for one hour.	1,200	5,000	5,000
61	<i>S. ærtrycke</i>	OB heated at 93°C. for one hour.	1,200	5,000	2,000

*Note.*—The figures present the redenominators of titres, i.e., the highest dilution of serum in which a partial (+) agglutination was observed by the naked eye according to the scheme described under Table II.

Table I summarizes the 'O' agglutination results obtained with three different strains of *B. paratyphosus A.* For comparison, results with a strain of *B. typhosus* (Ty Razmak A) and of *B. paratyphosus B* (OB, Schütze, 1930) are included, against which sera were prepared by identical procedure.

It is recognized that experiments on the small number of animals used will not be fully convincing but the constant results in successive tests make it possible to draw certain conclusions. It is seen that none of the strains of *B. paratyphosus A.*, whether injected live, killed, or phenolized, lead to the elaboration of 'O' antibody to the same degree as either *B. typhosus* or *B. paratyphosus B* similarly treated.

This is in accord with our previous finding (Bhatnagar, Freeman and Dhilon, *loc. cit.*) where, in an examination of over 500 sera of human subjects after T. A. B. inoculation, a similar divergence in the content of 'O' antibody was observed, titres against *B. typhosus* and *B. paratyphosus* B being much higher than against *B. paratyphosus* A.

The three strains of *B. paratyphosus* A, between themselves, show a marked difference in the production of 'O' antibody. The sera obtained with the *Mears* strain, whether used in the live state, heated, or carbolized, give a lower titre as compared with the other two strains. Whilst the highest titre obtained with *Mears* strain, when used in the live state for immunization, was 1 : 3,000, that with the other two strains was 1 : 5,000—a difference quite significant when the low agglutinogenic capacity of this organism is taken into consideration. When heated and carbolized suspensions were employed a similar discrepancy was found to exist.

It was carefully ascertained that all the strains were used in as smooth a state as possible by daily sub-culturing and by colony selection; in fact the colonies of strain *Mears* presented at all times a more smooth appearance than the other two strains and were completely stable in salt concentrations up to 6.8 per cent.

In order to determine whether the route of animal inoculation had any influence on low 'O' antibody production, the *Mears* strain was injected subcutaneously in identical doses and the antibody production compared with that obtained with strains of *B. typhosus* and *B. paratyphosus* B. The results are included in Table I and are found to be of the same order.

So far as these tests go they suggest that the *Mears* strain for use in vaccine is probably of lower antigenic value than other more recently isolated strains.

#### THE HEAT STABILITY OF SOMATIC 'O' ANTIGEN OF *B. paratyphosus* A.

The results incorporated in Table I go to show that when all the three strains of *B. paratyphosus* A, employed in these experiments, are exposed to a temperature of 93°C. for one hour, their agglutinogenic capacity is almost entirely lost, the titres obtained with a dose of  $1,200 \times 10^6$  organisms being 1 : 100 (*Mears*), 1 : 150 (*ASA*) and 1 : 200 (*RK*). This observation is of interest in view of the fact that [it] is generally accepted that the somatic antigens are highly heat stable. A definite difference in this respect is shown between *B. paratyphosus* A and the *B. typhosus* and *B. paratyphosus* B strains examined.

#### THE PRESENCE OF 'Vi' ANTIGEN IN CERTAIN STRAINS OF *B. paratyphosus* A.

Following the differences, noted above, in the agglutinogenic power of strain *Mears* on the one hand and the strains *ASA* and *RK* on the other, it was considered desirable to investigate whether Indian strains of *B. paratyphosus* A contained an antigen of the nature of the 'Vi' antigen of *B. typhosus*. During the course of these investigations attention was drawn to a paper by Felix and Pitt (1936) in which

they have demonstrated the presence of 'Vi' antigen in various *Salmonella* types by the use of acid treated bacteria. Felix, Krikorian and Reitler (1935) had previously expressed their inability to detect this antigen both in the freshly isolated strains and in stock cultures of paratyphoid strains by the same methods which led Felix and his co-workers to discover it in certain strains of *B. typhosus*.

As a routine all freshly isolated strains of *B. paratyphosus A* were examined on the lines adopted for demonstration of 'Vi' antigen in the case of *B. typhosus*. Strains of stock cultures were also included for comparison. Whilst the latter, including the strain *Mears*, at no time showed any evidence of the presence of 'Vi' antigen, one of the freshly isolated strains—*ASA*—gave indications of its presence. Its agglutination reactions against a pure 'O' serum are presented in Table II:—

TABLE II.

*Agglutination by pure 'O' serum.*

Serum.	Dilution.	AGGLUTINATION OF STRAINS OF <i>B. PARATYPHOSUS A.</i>					
		<i>ASA</i> GROWN ON LEMCO AGAR.				Mears.	Schott-müller.
		2ND DAY AFTER ISOLA-TION.		9th day.	16th day.		
		Living.	Heated at 60°C. for one hour.				
Pure 'O' serum.	100	+±	++±	++±	+++	+++	+++
	200	+	++±	+±	++±	++±	++±
	500	Tr.	++	+	++	++	++
	1,000	F. tr.	+±	Tr.	+±	+±	++
	2,000	—	+	—	+	+±	+±
	5,000	—	±	—	±	+	+
Virulence to mice ..		$\frac{1}{12}$	..		$\frac{1}{12}$	..	
Dose in millions of organisms.		100	..	..	500	..	..

*Note.*—The interpretation of agglutination is according to the following scheme:—

Observed with naked eye.	+++	Plentiful sediment, supernatant fluid clear.
	++	Plentiful sediment, supernatant fluid turbid.
	+	Small sediment, flakes in supernatant fluid.
Observed with 10 × lens.	±	Very small flakes in supernatant fluid.
	Tr.	Trace.
	F. tr.	Faint trace.



It will be seen that this organism possessed a well-marked resistance to 'O' agglutination on the second day after isolation as compared with two stock strains (Mears and Schottmuller). By the 16th day it had, however, become as agglutinable as the latter two organisms or its emulsion heated to 60°C. for one hour on the second day after isolation. That this phenomenon was not due to serum fastness or any related factor became evident by an interesting decrease in its virulence side by side with the loss of inagglutinability. The results of animal experiments, included in Table II, go to show that, while on the second day after isolation a dose of  $100 \times 10^6$  organisms killed 10 out of 12 mice, on the 16th day, during which time daily sub-culturing on lemco agar was resorted to, as many as  $500 \times 10^6$  organisms were required to kill 8 out of 12 mice experimented on.

The loss of inagglutinability and the consequent decrease in virulence are believed to be due to the unsuitability of ordinary laboratory media—lemco agar or fresh beef or horse-flesh agar—for the maintenance of an antigen which is far more labile than the 'H' antigen itself. Various other media, such as Douglas' trypticized heart-digest agar, have so far proved disappointing in our hands. Attention is, however, drawn to the ascitic agar prepared according to the method described by Kaufmann (1935), which is giving satisfactory results. We have not tried the peptone-salt agar medium recommended by Horgan (1936) very recently.

The same experience has been met with in the case of *B. typhosus* of known 'Vi' antigen content especially when it is grown on lemco agar or fresh beef agar, where the quality of beef is poor. Two strains of this organism from fatal cases, one isolated by us (S. B.) and the other (W.) obtained through the courtesy of the Professor of Pathology, King Edward Medical College, Lahore, proved more virulent than the most inagglutinable strain so far experimented on (Ty 2, Weil and Felix, 1920), but on cultivation on lemco agar both of these strains reverted to the intermediate type (Felix, Bhatnagar and Pitt, 1934) in as short a period as seven days. This may be one of the explanations why virulence tests applied to the freshly isolated strains of *B. typhosus* prove them to be of moderate virulence only. In any experiments, therefore, the medium on which the organisms are cultivated may be found to influence the retention of 'Vi' characters and the original status of a strain as of 'Vi' type may easily be lost.

#### THE AGGLUTINABILITY OF *B. paratyphosus* A.

Since the adverse effect of heat on the antigenic activity of *B. paratyphosus* A was noticed, it was considered desirable to determine whether the agglutinability of this organism suffered *pari passu* under similar circumstances. The agglutination of various strains after heating at 60°C., 70°C., and 93°C. for one hour was, therefore, tested against a pure 'O' serum. The results obtained with one of these strains namely OAK, an organism very sensitive to 'O' type of agglutination, are embodied in Table III. For comparison, agglutination against the living organism is also included.

A study of Table III and Table I taken together will indicate that the effect of heat on agglutinogenic capacity and agglutinability differs. While the power to induce antibody formation is almost lost with all the three strains experimented on,

when the organisms are heated to 93°C. for one hour, agglutinability on the other hand, though reduced, is quite considerable. In addition, it is seen from Table III that the live organisms are decidedly more sensitive to agglutination as compared with the same strain heated at various temperatures.

TABLE III.

*The agglutinability of B. paratyphosus A.*

Serum.	Dilution.	THE EFFECT OF HEAT ON AGGLUTINATION OF OAK STRAIN OF <i>B. paratyphosus A.</i>			
		Live.	Heated at 60°C.	Heated at 70°C.	Heated at 93°C.
No. 47	100	+++	+++	+++	++
	200	+++	++	++	++
	500	+++	++	+	+
	1,000	++	++	±	Tr.
	2,000	++	+	Tr.	—
	5,000	+	±	—	—
	10,000	—	—	—	—
No. 41	100	+++	+++	++	++
	200	+++	++	++	++
	500	++	++	+	+
	1,000	++	+	±	±
	2,000	+	±	Tr.	F. tr.
	5,000	±	—	—	—
	10,000	—	—	—	—

Note.—(1) Organisms heated for one hour.

(2) Readings taken after 24 hours; two hours at 37°C., then room temperature for 22 hours.

For interpretation see note under Table II.

## DISCUSSION.

The determination of the serological characters of strains of organisms incorporated in a T. A. B. vaccine is of first importance and has been especially emphasized by the studies of Grinnell (1932), and Perry, Findlay and Bensted (1933a, b;

1934a, b). Felix and his co-workers (Felix and Pitt, 1934; Felix, Bhatnagar and Pitt, 1934; Felix and Bhatnagar, 1935) have demonstrated the different serological complexes of different strains of the same organism. The practical application of these investigations is reflected in the replacement of standard laboratory strains, such as, *Rawling's* of *B. typhosus*, formerly used by many laboratories and which may have altered in antigenic constitution, by more recent strains of determined antigenic type for the purpose of vaccine manufacture.

All these studies have, however, been confined to *B. typhosus*. The paratyphoid strains forming part of the T. A. B. vaccine have not so far received investigation. The accumulated data of past few years' experience pointed to the fact that the practice of T. A. B. inoculation in a population under control did not help to reduce the incidence of paratyphoid A infection to the same degree as that of typhoid fever.

The investigation, as detailed above, shows that the *Mears* strain of *B. paratyphosus* A, used in the vaccine in a perfectly smooth state, fails to elaborate the 'O' antibody to the same degree as two other recently isolated strains included for comparison. Since the immunological significance of 'O' antibody has been well established in prophylaxis (for references see Topley, 1933), and the immunity is now recognized to depend partly on the presence of antibodies *per se* and partly on accompanying cellular changes which are intimately connected with antibody formation itself, this study on experimental animals, taken in conjunction with a similar experience in human beings (Bhatnagar, Freeman and Dhillon, *loc. cit.*), would strongly stress the desirability of enhancing the immunizing value of the present paratyphoid A component of the T. A. B. vaccine by the substitution of a recently isolated strain, of suitable serological complex, as practised for *B. typhosus*, in place of the older *Mears* strain.\*

A divergent effect of the thermic influence on the property of agglutinability and that of agglutininogenicity has been demonstrated in this communication. That the biological properties of a bacterial antigen may be affected differently by physical and chemical agents has been the experience of other observers also. Thus, Eisenberg and Volk (1902), while determining the nature of the union of antigen and antibody, concluded that heat and other agencies destroyed the agglutinophore and left the haptophore groups relatively unaltered. Felix and Pitt (*loc. cit.*) state that the agglutininogenic properties of both the 'Vi' and the 'O' antigen of *B. typhosus* are not impaired by treatment with alcohol, while the agglutinability by the 'Vi' antibody of alcohol-treated suspensions is almost annulled.

The loss of resistance to 'O' agglutination and the accompanying decrease in virulence of freshly isolated highly virulent strains, when grown on ordinary laboratory media, suggest the possibility that a labile factor of the same nature as the 'Vi' antigen of *B. typhosus* is responsible for this change. As any S→R variation was carefully guarded against, this study shows that the mere presence of smooth 'O' antigen does not connote the highest degree of virulence in paratyphoid A strains. Those organisms which possess this new factor should, therefore, be included in the preparation of a T. A. B. vaccine and optimum conditions aimed at for its maintenance.

\* We have been informed that, on the results of a comparison of strains, this procedure has been adopted by the Central Research Institute, Kasauli, in the preparation of their T. A. B. vaccine.

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### SUMMARY.

(1) The production of 'O' antibody by *B. paratyphosus A* falls significantly short in comparison with *B. typhosus* and *B. paratyphosus B* when immunization is carried out with live, heated or phenolized suspensions and by either the intravenous or the subcutaneous route.

(2) The *Mears* strain of *B. paratyphosus A* when compared with two recently isolated strains produces a much lower antigenic response as judged by the agglutinin titre.

(3) Certain strains of *B. paratyphosus A* are resistant to 'O' agglutination and are of high virulence. Their inclusion in a T. A. B. vaccine is advocated.

(4) Exposure to 93°C. for one hour almost annuls the agglutinogenic activity of *B. paratyphosus A*. Its agglutinability at this temperature, however, remains relatively unaltered.

(5) The unsuitability of ordinary laboratory media for the maintenance of high virulence in the typhoid-paratyphoid group of organisms is demonstrated.

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## ADSORPTION OF ANTIGENS BY ANTIBODIES OR VICE VERSA.

### Part III.

#### THE EFFECT OF ELECTROLYTES ON THE RATE OF FLOCCULATION OF TOXIN-ANTITOXIN MIXTURES OF DIPHTHERIA AND TETANUS.

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SINCE Ramon's (1922*a* and *b*) observation that, of a series of mixtures of diphtheria toxin and antitoxin, the neutral or the balanced mixture flocculates more quickly than the others, a large number of papers on this flocculation reaction have been published. The influence of the nature of the toxin and the antitoxin on the rate of flocculation has been studied by Glenney *et al.* (1925). It has been found that the rate depends markedly on the potency of the toxin. As a rule, the more potent the toxin, the quicker was the flocculation obtained with a particular antitoxin. Again, using a given toxin the time of flocculation varied with different samples of antitoxin.

The reaction of the medium has also an important effect on the rate of flocculation. Schmidt (1930*a* and *b*) observed that it (the rate of flocculation) does not alter appreciably between the range of pH 5.5 and pH 8.0. At pH 4.49 and pH 9.5 the rate is very slow, while at pH 10.0 flocculation does not occur. The effect of salts on the rate of flocculation has been found to depend on their concentration. In low concentrations they usually favour the flocculation, while at high concentrations they retard it. Thus, Schmidt (1930*b*) records that in high concentrations the anions  $\text{ClO}_4$ ,  $\text{SCN}$ ,  $\text{ClO}_3$ ,  $\text{NO}_3$ ,  $\text{Br}$ ,  $\text{IO}_3$ ,  $\text{SO}_4$ ,  $\text{Cl}$ ,  $\text{NO}_2$  inhibit flocculation, and arranging them in order of their inhibiting power he obtained the following series  $\text{ClO}_4 > \text{SCN} > \text{ClO}_3 > \text{NO}_3 > \text{Br} > \text{IO}_3 > \text{SO}_4 > \text{Cl} > \text{NO}_2$ .

Since the process of peptization is the reverse of that of flocculation it is to be expected that high concentration of salts will favour the peptization of the floccules.

Marrack and Smith (1930) noticed that diphtheria-antitoxin floccules were peptized by high concentration of salts containing the anions salicylate, iodide, thiocyanate, bromide and nitrate. The order of their peptizing power has been found to be salicylate > I > SCN > Br > NO<sub>3</sub>.

It is to be noted, however, that although unconcentrated diphtheria antitoxin when mixed with the right quantity of the toxin flocculates easily, yet the concentrated antitoxin freed from serum albumin and the so-called euglobulin fraction does not flocculate on mixing with the toxin (*cf.* Ramon, 1922b). This failure of flocculation might lead one to suppose that concentrated diphtheria antitoxin does not combine with the toxin under ordinary laboratory conditions. Eagle (1935) has, however, brought forward evidence which indicates that purified diphtheria antitoxin does combine with the toxin *in vitro*. An analysis of these facts leads to the conclusion that the diphtheria toxin-antitoxin reaction occurs in at least two stages.

The first stage consists in the adsorption of the particles of toxin by those of the antitoxin leading to the formation of neutral particles containing both toxin and antitoxin. These neutral particles we may term the 'complex primary particles', as they serve as units in the subsequent process of flocculation. The second stage consists in the aggregation of the 'complex primary particles' (formed in the first stage of reaction) into bigger ones, which finally form floccules. Thus the failure to obtain Ramon's flocculation test in purified diphtheria antitoxin solution is to be attributed to the fact that in such solution the aggregation of the 'complex primary particles' is prevented by some inhibiting factors. The present work was undertaken with a view to find out how far by a suitable choice of electrolytes and toxin, and by the adjustment of the dilution of the toxin and the antitoxin, it is possible to overcome the effect of the inhibiting factors. The results so far obtained are recorded in this paper.

## EXPERIMENT.

### *Determination of flocculating power of a few electrolytes and non-electrolytes.*

In the first place the effect of a number of substances on the rate of flocculation of mixtures of diphtheria toxin and (unconcentrated) antitoxin was studied. This enabled us to select a few electrolytes of high flocculating power and also gave us an idea of the optimum concentrations in which they should be employed in subsequent experiments.

To determine the flocculating power of a substance, a stock solution of it was prepared. The toxin and the antitoxin were suitably diluted with this stock solution in separate flasks. A fixed quantity of the diluted toxin (2 c.c.) was delivered into a series of glass tubes of uniform diameters. Different quantities of the diluted antitoxin were then added to the tubes and their volumes adjusted to a definite amount by adding the requisite quantities of the stock solution. The tubes were shaken and placed in a bath maintained at 50°C. and the time required for the first appearance of visible flocculation noted. For each series of experiments all the factors except the nature of the flocculating agent were maintained,

as far as possible, constant so that the results are fairly comparable among themselves. Some of the data obtained are recorded in Table I :—

TABLE I.

*Concentration of the diluted toxin—1/5th of the original toxin.*  
*Concentration of the diluted antitoxin—1/30th of the original antitoxin.*

$K_f$  = the time of commencement of flocculation.

Substance used.	Molar concentration.	$K_f$ in minutes.
Glucose .. .. .	0·33	Infinite.
Urea .. .. .	0·17	"
Sodium chloride .. .. .	0·15	180
Sodium oxalate .. .. .	0·08	120
Sodium phosphate ( $Na_2HPO_4$ ) .. .. .	0·07	120
Sodium citrate .. .. .	0·044	90
Sodium citrate .. .. .	0·132	45

The data recorded in the above table show that taking sodium chloride as the standard for comparison solutions of urea and glucose inhibit flocculation, while sodium citrate has the highest flocculating power.

If the four electrolytes are arranged in the order of their flocculating power then the following series is obtained :—

Citrate > phosphate > oxalate > chloride.

It was, therefore, decided to try the effect of citrate and phosphate solutions of different concentrations on mixtures of diphtheria toxin and its concentrated antitoxin.

#### *Preparation of concentrated diphtheria antitoxin and study of its flocculation reaction.*

The major part of the diphtheria antitoxin contained in immunized horse serum is precipitated with the pseudoglobulin fraction.

The antitoxic serum was first diluted with water and then sufficient quantity of saturated ammonium sulphate solution is added to it so as to make the solution 30 per cent saturated with respect to ammonium sulphate. This precipitates the euglobulin. It was filtered and to the filtrate equal volume of 70 per cent saturated ammonium sulphate added. This makes the whole solution half saturated with respect to ammonium sulphate. The precipitate consisting of the pseudoglobulin fraction is separated by filtration and dialysed in a cellophane bag against water in a cold chamber. When it is free from ammonium sulphate it is diluted to five times its original volume with distilled water and its pH adjusted to 5·6 and the requisite quantity of tricresol added. After keeping in a cold chamber for about a week, it was filtered through a Seitz filter and used for our experiments. The remaining experimental procedure was the

same as described in the previous section. The results are recorded in Table II. From these data it will be noticed that using sodium citrate and sodium phosphate as flocculating agents concentrated diphtheria antitoxin gives flocculation with its antigen.

TABLE II.

*Concentration of the diluted toxin—1/5th of the original toxin.*  
*Concentration of the diluted antitoxin—1/30th of the original antitoxin.*

Electrolyte used.	Molar concentration.	Time of commencement of flocculation in minutes.
Sodium citrate .. .. {	0.06	120
	0.12	90
	0.17	45
Sodium phosphate ( $\text{Na}_2\text{HPO}_4$ ) .. {	0.14	100
	0.21	80

The effect of the potency of the toxin on the rate of flocculation was also tried. The results are to be found in Table III. These data show that with a given electrolyte and a given sample of concentrated antitoxin the rate of flocculation depends markedly on the potency of the toxin. In fact if the potency of the toxin is less than 7  $\text{L}_f$  units per c.c. there is hardly any flocculation within a reasonable period of time.

TABLE III.

*Strength of the diluted toxin—1/5th of the original toxin.*  
*Strength of the diluted antitoxin—1/20th of the original antitoxin.*  
*Strength of the sodium citrate solution—3.5 per cent.*

Number of concentrated antitoxin sample.	Number of toxin sample.	Potency of toxin in $\text{L}_f$ units per c.c.	$\text{K}_f$ in minutes.
1 {	A	30	45
	B	16.5	120
	C	7.2	190
2 {	A	30	50
	B	16.5	128
	C	7.2	260



*The agreement between the potency of the concentrated antitoxin (diphtheria) determined by the flocculation test and that determined by animal experiment.*

Having observed that purified diphtheria antitoxin gives flocculation test with its antigen in presence of sodium citrate or phosphate, we tried to determine how far this test can be used as a means of measuring the potency of the antitoxin. For this purpose, simultaneous flocculation tests and animal experiments were carried out using always the same sample of toxin. This toxin sample was standardized with reference to a standard antitoxin shortly before this series of experiments were started.

The results are recorded in Table IV. It will be noticed that the agreement between the results obtained by the two methods is fair.

TABLE IV.

*Flocculation of mixtures of concentrated tetanus antitoxin with its antigen.*

Sample number of antitoxin.	Units per c.c. of concentrated antitoxin found by flocculation test.	Units per c.c. of concentrated antitoxin found by animal experiment.
1	960	1,000
2	1,225	1,200
3	1,650	1,600
4	1,680	1,800
5	1,445	1,500
6	1,750	1,800
7	2,080	2,000
8	1,700	1,800
9	1,200	1,200

*Flocculation of concentrated tetanus antitoxin by tetanus toxin.*

In some preliminary experiments with a mixture of fresh antitoxic serum and tetanus toxin, we obtained flocculation in presence of 3.5 per cent citrate solution. Frequently, however, two zones of flocculation at two different toxin-antitoxin ratios were obtained.

Animal experiments showed that only one of these zones corresponds with the balanced mixture of tetanus toxin and antitoxin; the other zone is non-specific. It was thought that purification and concentration of the antitoxin might eliminate the non-specific reaction as it was comparatively weak and gives only the specific one. Some experiments were, therefore, made with concentrated tetanus antitoxin. The concentration was effected in exactly the same way in which diphtheria antitoxic serum was concentrated. The results of flocculation test along with those of animal tests are recorded in Table V. The potency of the antitoxin is expressed in the table in American units. It will be noticed that there is fair agreement between the two sets of data. It is needless to add that these results are as yet of a preliminary nature but they appear to be of sufficient significance to be incorporated in this paper. Further work in this line is in progress.

TABLE V.

*Strength of the solution—3·5 per cent.*

Sample number of antitoxin.	Units per c.c. obtained by flocculation test.	Units per c.c. obtained by animal test.
1	1,550	1,500
2	1,800	2,000
3	1,280	1,400
4	1,050	950
5	1,450	1,600
6	1,650	1,800
7	2,166	2,250
8	1,790	1,850

It may be mentioned that in none of the above experiments the non-specific reaction was noticeable.

## SUMMARY.

1. The effect of a few substances on the rate of flocculation of diphtheria toxin-antitoxin mixture has been determined. The order of flocculating power of the sodium salts of electrolytes used was citrate > phosphate > oxalate > chloride.

Urea and glucose solutions do not produce flocculation under the conditions of the experiment.

2. In presence of sodium citrate or disodium hydrogen phosphate flocculation is obtained with purified and concentrated diphtheria antitoxin. The rate of flocculation has been found to depend on the potency of the toxin and on the concentration of the electrolytes used.

3. In presence of citrate of sodium purified and concentrated tetanus antitoxin also gives flocculation. The data so far obtained show that the balanced mixture flocculates most quickly compared with the other mixtures.

In conclusion, we thank very warmly the authorities of the Bengal Immunity for offering excellent facilities for carrying out this piece of work.

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## CANCER IN INDIA.

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OUR last paper on Cancer in India, published in this *Journal* in July 1935, embodied results of a survey covering the provinces of the Punjab, Delhi, United Provinces, and Bihar & Orissa. This paper presents data since collected from Madras and Burma.

A much larger number of autopsy records were available for examination in this survey (6,416) than was the case in Northern India (1,234) where popular prejudice greatly reduces the number of post-mortem examinations by pathologists. The records under review dealt with post-mortem examinations carried out by pathologists to the Medical College, Rangoon, and the Medical College, Madras. The latter included 404 examinations carried out at the Raipurum Medical School, Madras.

Of the 6,416 post-mortem examinations made, a diagnosis of malignant disease was arrived at in 260, showing an incidence of 1 in 24.7. Of the 2,454 examinations made in Rangoon, malignant disease was detected in 97 and of the 3,962 carried out in Madras 163 showed presence of malignant disease. This gives an incidence of 1 in 25.3 for Rangoon and 1 in 24.3 for Madras. It must not be inferred from this that the incidence of cancer as judged from autopsy records is practically the same amongst Burmans and Madraseds. Rangoon has a mixed population. This mixture is reflected in the record of 2,454 post-mortem examinations under discussion. One thousand five hundred and ninety-four of these examinations were carried out on Indian subjects and 860 on Burmans. The incidence of malignant disease in the former group was 1 in 39 and in the latter 1 in 16.

The proportion of carcinoma to sarcoma in the Rangoon figures was 3·5 : 1 and in Madras 6·85 : 1 ; with the Rangoon and Madras figures taken together the proportion is 5 : 1.

The site of maximum incidence is the gastro-intestinal tract, taken together with liver and pancreas. In Rangoon this site accounts for 50 per cent of the cases—the incidence amongst Indians and Burmans being practically the same. In Madras, the figure is even higher (62·1 per cent). The combined figure is 58 per cent. In Madras the site second in order of frequency is buccal (14·6 per cent) and cancer of the female genital tract (5·1 per cent) comes third. In Rangoon, cancer of the female genital tract (24·3 per cent) comes second and buccal cancer (9·5 per cent) occupies the third place.

Two hundred and sixty deaths from malignant disease, of which 205 were due to cancer, is too small a figure to use for purposes of comparison. Table II, indicating proportional frequency of cancer as affecting different sites in the human body, is therefore of very limited significance as compared with the English data quoted.

The incidence of cancer was also ascertained from records of medico-legal autopsies performed in Rangoon from 1930 to 1934. Records of 3,150 examinations were studied relating to deaths from violence, poisoning, or accidents. Malignant disease was discovered in 17 cases in this series. It was not the cause of death but discovery incidental to the investigation made. Of the 17, sixteen were cases of cancer and one of sarcoma—a male. Of the cases of cancer 12 were male and 4 female. The order of frequency according to sites affected was gastro-intestinal tract including the liver (six cases), buccal (four cases), female genital tract (two cases), and miscellaneous (four cases).

Records of morbid histology show that of a series of 11,801 specimens examined in Madras 2,236 were diagnosed malignant and that in Rangoon of 2,814 specimens examined a diagnosis of malignant disease was returned in 741. In other words one out of every five specimens in Madras and one out of four in Rangoon were diagnosed to be histologically malignant.

A study of the regional incidence based on the records of Madras and Rangoon taken together (Table VII) shows that cancer of the female genital tract (19·7 per cent) represents the site of maximum incidence. Buccal cancer (18·5 per cent) comes next and that cancer of the breast and skin both compete for the third place (13 per cent). Cancer of the penis comes next in order of frequency (10·2 per cent) and then the gastro-intestinal tract and liver (5·2 per cent).

Viewing this incidence separately in Madras and Rangoon the order of frequency is found to be the same in either town for the first two sites, viz., female genital tract and buccal. The third place, however, is occupied in Madras by cancer of the skin and the fourth by cancer of the breast. In Rangoon it is vice versa.

Considering the distribution of regional incidence for the two sexes (Tables VIII and IX) we find that buccal cancer shows a disproportionately high incidence in men (27·3 per cent) as compared with women (10·3 per cent). The disproportion is evident to nearly the same extent when the figures for Madras and Rangoon are considered separately. The communal distribution of this form of cancer shows its highest incidence amongst Mohammedan males both in Rangoon (50 per cent)

and Madras (48·9 per cent). In Rangoon, Hindu males show an incidence of 39·5 per cent and Burmese males of 20·3 per cent. The figures of incidence of buccal cancer—much lower for women—show the same relative communal distribution.

Incidence of cancer of the gastro-intestinal tract including the liver falls heavier on the male (7·2 per cent) than on the female (2·6 per cent). This disproportion is evident both in the Madras and Rangoon figures. Communally the incidence is a little higher amongst Hindu males in Madras and Mohammedan males in Rangoon.

So far as cancer of the skin is concerned, the incidence is again higher in the male (16·5 per cent) and lower in the female (10·4 per cent). Rangoon and Madras figures also bear out this observation separately. Communally both Mohammedan males and females show higher incidence of this form of cancer, and in Rangoon the non-Indian population, particularly male, more than the Indian.

Cancer of the breast showed a higher incidence in Rangoon (27·8 per cent) than in Madras (25·4 per cent). Its incidence in Rangoon was nearly twice as high amongst non-Indian women as amongst Indian.

Cancer of the female genital tract—mostly carcinoma of the cervix—showed (Table IXa) its highest incidence amongst Hindu women in Rangoon (47·2 per cent), Burmese women came next (41·7 per cent); Mohammedan women showed 33·3 per cent. In Madras too the highest incidence was amongst Hindu women (42·1 per cent) and lowest amongst Mohammedans (26·7 per cent).

As regards penile cancer (Table VIII), this is practically confined to the non-circumcized communities. The Rangoon figures show an incidence of 14·8 per cent, Madras of 22·4 per cent. In Rangoon the highest incidence was amongst the Burmese with 24·7 per cent, Hindus came next with 11·8 per cent. In Madras figures for Hindus were highest with 24·5 per cent, for other non-Mohammedan communities 18·5 per cent.

Clinical records of cases treated in the in-patients' departments of teaching hospitals in Madras and Rangoon showed (Table X) that out of a total of 222,442 patients treated 5,776 were diagnosed to be suffering from malignant disease. Out of a total of 163,470 in-patients Madras showed 4,922 cases of malignant disease and Rangoon out of 58,972 in-patients 854.

In the Rangoon General Hospital the incidence worked out to be 1 in 69; in the Madras General Hospital 1 in 25; in the Madras Maternity Hospital 1 in 28; in the Raipurum Medical School Hospital 1 in 89.

The age of highest incidence of cancer according to records of autopsies, morbid histology, and patients treated indoors in the Rangoon and Madras hospitals (Tables III, V and XII) is between 40 and 50. The incidence is fairly high even between 30 and 40. Sarcomata shows high incidence between 20 and 40, although noticeable frequency begins right from infancy.

The records show (Table XVII) that, in Madras, buccal cancer shows the highest incidence (37·9 per cent). Cancer of the female genital tract comes next (31·08 per cent) and cancer of the gastro-intestinal tract and liver occupies the third place. In Rangoon cancer of the female genital tract (30·7 per cent) occupies the first place, gastro-intestinal tract (21·7 per cent) the second and buccal cancer (13·9 per cent) comes third. There is not much difference between the percentage

incidence for breast in Madras (7.25 per cent) and Rangoon (8.8 per cent) or of penile cancer—Madras 5.45 per cent, Rangoon 5.1 per cent. These figures represent incidence on the two sexes taken together.

Viewing percentage incidence separately for the two sexes (Table XVI) one finds that the high incidence of buccal cancer in Madras becomes remarkably high for the male (60.15 per cent) against female (17.9 per cent). Same is true of Rangoon (Table XVIb)—male 22.9 per cent, female 5.6 per cent (Table XVIc). Cancer of the gastro-intestinal tract also shows heavier incidence on the male. In Madras the incidence was 15.55 per cent for the male and 4.3 per cent for the female. In Rangoon it was 36.1 per cent for the male and 8.45 per cent for the female. Similarly is the case with cancer of the skin; in Madras 2.95 per cent for the male and 0.9 per cent for the female; in Rangoon 5.4 per cent for the male and 0.7 per cent for the female.

Considering the same incidence by communities one finds (Table XVI) that buccal cancer shows the highest incidence in the Mohammedan male both in Madras (70 per cent) and Rangoon (Table XVIb) (37.8 per cent). In Rangoon, Hindu males come next with 35.5 per cent and Burmese last with 13 per cent. Incidence amongst women is in the same communal order.

As regards cancer of the gastro-intestinal tract the Rangoon figures again give (Table XVIb) the Mohammedan male (39.1 per cent) a lead over the Hindu (31.3 per cent) and Burmese (36.5 per cent) males. Males of communities other than these three showed an even higher incidence (41.3 per cent). The total incidence over this group was, however, so small that much importance cannot be given to this percentage incidence. Burmese women showed this form of cancer in a higher percentage than women of other communities, but here again the total number dealt with was small.

Cancer of the skin showed (Table XVIb) a higher incidence on the Burmese (9.9 per cent) in Rangoon than on the Hindus (3.6 per cent).

Practically the entire incidence of penile cancer was on the uncircumcized (Tables XVI and XVIb). Taking cancer incidence on the male, penile cancer represents 10.8 per cent of the total in Rangoon and 10.55 per cent in Madras. In Rangoon the Burmese lead with 17.7 per cent, Hindus come next with 7.6 per cent and other non-Mohammedan males with 4.8 per cent. In Madras the Hindus show 12.5 per cent and other non-Mohammedan males 5.1 per cent.

Cancer of the female genital tract (Table XVIa and c) has a practically similar incidence in Rangoon (58.5 per cent) and Madras (60.9 per cent). The preponderating incidence is on Hindu women both in Rangoon (70.6 per cent) and Madras (62.3 per cent); Burmese women in Rangoon with 57.5 per cent come next.

Cancer of the breast shows (Table XVIa and c) a higher incidence in Rangoon (16.9 per cent) than in Madras (12.05 per cent) and in the former town higher in Burmese women (20 per cent) than amongst Hindu (4.4 per cent).

The ratio of sarcoma to carcinoma in the Rangoon General Hospital was 1 : 7; in the Madras General Hospital 1 : 8.4; in the Raipurum Medical School, Madras, 1 : 6.6.

Table XIX shows the number of cases of malignant disease returned by provincial hospitals with indoor accommodation. Our investigation convinced us



that these returns were not as accurate as they could be. In Burma, the proportion of cases of malignant disease to total number of in-patients treated was 1 : 164 and in Madras 1 : 89.

#### DISCUSSION.

The foregoing data and those set forth in our previous paper (Nath and Grewal, 1935) offer only a very rough measure of the incidence of cancer in India. In the absence of proper vital statistics, one has to seek indices of prevalence from sources such as we have utilized. Out of a thousand deaths from all causes in England, cancer accounted for 132 in the year 1934 (Ministry of Health, 1934). In the 6,416 pathological examinations considered in this paper cancer accounts for 40·5 deaths per thousand. If mortality, minus deaths from infectious diseases which do not prevail in England but which raise our death rate much above the English level, were considered, the proportion of cancer deaths will not be found to differ much from that obtaining in England or other Western countries.

In England the crude death rate from cancer per million persons living was 1,563 in 1934 (Ministry of Health, *loc. cit.*). We have no data for offering such accurate information, but a consideration of the incidence of cancer among 3,150 persons who met with sudden death through violence, poisoning, or accident and who but for such an end could constitute a random sample of the living population may prove interesting. If cancer prevailed in the general population at the same rate as in this group (which of course it cannot, since the group was not diluted by subjects of insusceptible age), incidence of cancer would be 3·4 times as high as in England. This is an extremely crude, perhaps ridiculous, comparison but it is offered as giving some support to our general impression that the incidence of malignant disease in the East is not far behind that in the West.

Both the pathological and medico-legal autopsy records show that the commonest site of cancer is the gastro-intestinal tract taken together with the liver and pancreas. This was also the conclusion offered by autopsy material reviewed in our last paper (Nath and Grewal, *loc. cit.*) and represents the site of maximum incidence particularly in the male almost all over the world.

The incidence was much higher in Madras (63·4 per cent) than in Rangoon (50 per cent). According to clinical records of all the provinces surveyed the prevalence of buccal cancer stands highest in Madras and there is no doubt that the same is true of peptic ulcer. Whatever the rights of the ulcer-cancer controversy regarding the stomach may be from an academic point of view, epidemiologically the association of the highest peptic ulcer rate with that of the highest incidence of the cancer of the gastro-intestinal tract and also of buccal cancer in one and the same province cannot be devoid of significance. The preponderating incidence of all three conditions is on the male sex.

Buccal cancer is a lesion of an exposed site and consequently not difficult of diagnosis. The incidence figures from all types of records, therefore, support one another and confirm buccal as a site of high frequency. The same is true of cancer of the cervix—which represents, so far as cancer in women is concerned, the site of maximum incidence with us as well as most other countries. Same was the case in England until quite recently, but birth control and scientific care of parturition and higher level of personal hygiene have now given cancer of this site a position subordinate to that of cancer of the breast.

As a site of maximum incidence of cancer the position of the gastro-intestinal tract, which is not an easily accessible site for purposes of diagnosis, rests, however, on the indubitable evidence of post-mortem examinations. That the histology and clinical records do not assign it a position of importance is a fact of no consequence. Scarcity of material for biopsies from such inaccessible sites and errors in diagnosis arising from the supposed rarity of cancer in India would explain the comparatively smaller figures of incidence of cancer of the gastro-intestinal tract under these heads.

The Indian population in Rangoon is drawn mostly from the Madras Presidency. Ethnologically, therefore, the people amongst whom the incidence of cancer was surveyed in this paper were Dravidian or Burmese—different to the people in Northern India, the locus of the last survey. It seems common social usages, personal habits and hygiene, more than race, determine a uniformity of incidence of cancer. For example, the incidence of cancer of the female genital tract—mainly the cervix—falls heaviest and at an earlier age period on the Hindu women in Madras as well as Rangoon, just as it did in Northern India—Burmese women come next and Mohammedan last. Early marriage, the trauma of frequent confinements, attendant focal sepsis, and, in the majority, chronic malnutrition must have an ætiological bearing on the frequency of cancer of this site amongst Hindu women.

Similarly, buccal cancer which showed the highest incidence amongst Mohammedan males in Northern India shows high incidence in Madras and Rangoon. Lack of oral hygiene, chewing *pan* reinforced with lime and tobacco and retention of the quid over long periods causing chronic irritation and abrasion of the buccal mucosa must be responsible for this relatively higher incidence. The following table from page 98 of the Chief Medical Officer's report for 1934 (Ministry of Health, *loc. cit.*) will afford interesting comparison :—

International nomenclature number.	Site.	MALE.		FEMALE.	
		Total deaths from cancer.	Deaths per thousand.	Total deaths from cancer.	Deaths per thousand.
45	Buccal cancer ..	2,812	98	537	16
46	Cancer of gastro- intestinal tract, liver and pan- creas.	17,704	614	15,958	489
	Uterus ..	..	..	4,313	132
	Penis ..	156	5	..	..
	Skin ..	618	21	500	15
	Breast ..	55	2	6,551	200

In Rangoon, cancer of the breast, skin, and penis showed relatively higher incidence in the Burmese. Benign papillomata of the penis are of relatively frequent occurrence in Rangoon. This may be due to the rigidity of standards prevailing in that centre in the matter of diagnosis of penile cancer. In addition to the usual histological criteria of malignancy, the presence of metastases and clinical signs are also ascertained; a diagnosis based on a constellation of all these features will be incontrovertible, but some early cases escape detection under these circumstances. The difficulty of labelling cancer of the penis on evidence other than histological will be illustrated by the following quotation from Willis (1935): 'Leighton (1932) stated that in carcinoma of the penis the inguinal glands are affected early but Kuttner (1900) and Morson (1930) found that the early enlargement of these glands was inflammatory only and that metastasis was long delayed and relatively infrequent; 32 per cent according to Kuttner. This author has shown also that the initial metastasis from penile cancer may occur in the pelvic lymph glands, while the inguinal glands remain unaffected. According to Bamey (1907) penile cancer yields metastasis in 60 per cent of cases but these appear usually late'.

Whatever the nature and importance of a common general factor possibly governing the causation of cancer may be, one cannot under-estimate the significance of the obvious and controllable carcinogenic factor of irritation as operating on particular sites like the mouth, the cervix uteri, the skin, and probably the gastrointestinal tract.

Dr. Stevenson studied the incidence of cancer in England by dividing the population into five classes on the basis of economic and educational standing and has brought out the significant fact that, so far as cancer of the exposed sites is concerned, its incidence in the most highly placed group Class I is nearly two-fifths of that of Class V (Annual Report of the Registrar-General, 1921).

It would be difficult in any country to bring the economic level of the population at large to Class I level but it is possible through education and public health propaganda to mitigate the evil effects of social customs and personal habits which promote the incidence of cancer.

The collection of the foregoing data was made possible only through the kind help of the superintendents and pathologists of the teaching hospitals visited and we express our grateful thanks to them.

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TABLE I.

*Incidence of malignant disease as shown in the autopsy records of the following institutions.*

Name of institution.	Total number of autopsies performed.	Total number diagnosed to have malignant disease.	Terratoma.	Carcinoma.	Sarcoma.	Endothelioma.	Hodgkin's disease.	Ratio of sarcoma to carcinoma.	Ratio of malignant disease to total number of autopsies.
Medical College, Rangoon.	2,454	97	1	74	21	1	..	1 : 3.5	1 : 25
Medical College, Madras.	3,558	154	1	131	18	2	2	1 : 7.3	1 : 23
Raipuram Medical School, Madras.	404	9	..	6	2	1	..	1 : 3	1 : 45
Total for above-mentioned institutions.	6,416	260	2	211	41	4	2	1 : 5.1	1 : 24.7
Medico-legal post-mortems, Rangoon.	3,150	17	..	16	1	..	..	1 : 16	1 : 185.5.3 per 1,000 post-mortems.

TABLE II.

*Incidence of malignant disease and regional distribution of cancer according to communities as ascertained from post-mortem records from 1909-35 of the pathological department of the Medical College, Madras.*

Name of community.	CANCER.								SARCOMA.			Teratoma.	Endothelioma.	Hodgkin's disease.	Total number of autopsies.	Ratio of malignant disease to total number of autopsies.
	Female genitals.	Penis.	Breast.	Gastro-intestinal tract and liver.	Buccal.	Skin.	Miscellaneous.	Rodent ulcer.	TOTAL.	Hard.	Soft.					
Hindu males ..	..	1	..	71	1	1	14	..	98	1	10	11	..	2	2,388	1 : 22
Hindu females ..	6	..	2	5	6	1	..	..	20	..	2	2	..	..	621	1 : 31
Mohammedan males ..	..	..	..	1	1	..	..	..	2	..	1	1	..	..	82	1 : 27
Mohammedan females ..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	12	..
Other males ..	..	..	..	4	1	..	1	..	6	..	3	3	..	..	288	1 : 32
Other females ..	1	..	1	2	1	..	1	1	7	1	..	1	1	..	71	1 : 8
Unknown ..	..	..	..	3	1	..	..	..	4	..	..	..	..	..	96	1 : 16
TOTALS ..	7	1	3	86	11	2	16	1	137	2	16	18	1	2	3,558	1 : 25

TABLE IIa.

*Incidence of malignant disease and regional distribution of cancer according to religion and sex as ascertained from post-mortem records from 1926-35 of the pathological department of the Medical College, Rangoon.*

Religion and sex.	CANCER.								SARCOMA.			Hodgkin's disease.	Teratoma.	Endothelioma.	Number of post-mortems performed.
	Female genitals.	Penis.	Breast.	Gastro-intestinal tract and liver.	Buccal.	Skin.	Miscellaneous.	Rodent ulcer.	Total.	Hard.	Soft.	Total.			
Hindu males	..	2	..	13	4	..	30	..	22	1	8	9	..	..	..
Hindu females	4	..	1	..	..	..	..	..	5	..	..	..	..	..	Indians 1,594.
Mohammedan males	..	..	..	1	..	..	..	..	1	..	..	..	..	..	Burmese 860.
Mohammedan females	..	..	..	..	..	..	..	..	..	..	..	..	..	..	Rates of malignant disease in Indians 1: 39, in Burmese 1: 16.
Burmese males	..	..	..	17	1	..	5	..	23	1	7	8	1	..	..
Burmese females	14	..	..	4	1	..	1	..	20	..	3	3	..	..	..
Other males	..	..	..	2	1	..	..	..	3	..	1	1	..	..	..
Other females	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
TOTALS	18	5	1	37	7	..	36	..	74	2	19	21	1	1	..

TABLE III.

*Incidence of malignant disease according to age as determined from post-mortem records of the Medical College, Rangoon, Medical College, Madras, and Raipurum Medical School, Madras.*

Age.	CARCINOMA.								SARCOMA.			Teratoma.	Endothelioma.	Hodgkin's disease.
	Female genitals.	Penis.	Breast.	Gastro-intestinal tract and liver.	Buccal.	Skin.	Miscellaneous.	Rodent ulcer.	Total.	Hard.	Soft.	Total.		
1-5	..	..	..	..	..	..	..	..	..	..	..	..	..	..
6-10	..	..	..	1	..	..	..	..	1	..	..	..	..	1
11-15	..	..	..	2	..	..	1	..	4	2	..	2	..	..
16-20	..	..	..	8	1	..	..	..	14	..	6	6	..	..
21-25	4	..	..	11	1	1	3	..	20	1	8	9	..	1
26-30	2	2	1	14	3	1	6	..	29	1	4	5	1	..
31-35	..	..	..	19	3	..	1	..	27	1	7	4	..	..
36-40	..	1	..	19	5	..	1	..	30	..	4	7	..	..
41-45	..	..	..	20	8	..	4	..	38	..	3	3	2	..
46-50	5	..	3	9	1	..	3	..	14	..	3	1	..	..
51-55	1	..	..	3	2	..	1	..	15	..	1	..	..	..
56-60	3	..	..	3	..	..	2	..	5	..	..	..	..	..
61-65	..	..	..	..	..	..	1	..	6	..	..	..	..	..
66-70	..	..	..	..	..	..	..	1	..	..	..	..	..	..
71-75	..	..	..	..	1	..	..	..	..	..	..	..	..	..
76-80	..	..	..	..	..	..	..	..	..	..	..	..	..	..
Above 80	..	..	..	6	1	..	..	..	7	..	..	..	..	..
Age not known ..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
TOTALS ..	25	3	4	124	27	2	25	1	211	4	37	41	4	2

TABLE IV.

*Incidence of malignant disease as based on morbid histology records of the following institutions.*

Name of institution.	CARCINOMA.							SARCOMA.			Total section.					
	Female genitals.	Penis.	Breast.	Gastro-intestinal tract and liver.	Buccal.	Skin.	Miscellaneous.	Rodent ulcer.	Total.	Hard.		Soft.	Total.			
Medical College, Rangoon.	122	41	86	21	114	51	127	11	573	29	77	106	20	25	17	2,814
Medical College, Madras.	277	123	144	75	221	180	243	8	1,271	78	249	327	94	55	17	11,801
Raipurum Medical School, Madras.	30	58	54	17	68	53	42	5	327	28	91	119	15	9	2	..
TOTALS ..	429	222	284	113	403	284	412	24	2,171	135	417	552	129	89	36	..



TABLE V.

Showing distribution of carcinoma according to age and site and of sarcoma according to age alone as gathered from pathological histology records of the Medical Colleges, Rangoon and Madras. and Medical School, Raipurum.

Age.	CARCINOMA.								Sarcoma.	Teratoma.	Endothelioma.	Hodgkin's disease.
	Female genitals.	Penis.	Breast.	Gastro-intestinal tract and liver.	Buccal.	Skin.	Miscellaneous.	Rodent ulcer.	TOTAL.			
1-5 ..	..	..	..	..	..	..	6	..	9	18	4	1
6-10 ..	..	1	..	..	..	3	12	..	2	25	6	5
11-15 ..	..	..	..	..	1	6	12	..	18	21	4	3
16-20 ..	..	3	2	2	2	10	24	..	38	39	13	4
21-25 ..	29	7	4	2	18	6	24	..	94	42	14	5
26-30 ..	47	19	26	14	36	24	44	1	211	70	10	3
31-35 ..	56	23	21	13	48	21	35	..	217	50	20	1
36-40 ..	65	23	51	17	65	38	58	2	322	71	14	2
41-45 ..	61	23	26	9	42	30	32	..	223	47	8	3
46-50 ..	68	34	54	16	44	41	41	1	299	45	9	3
51-55 ..	24	12	34	12	23	16	28	4	153	27	7	0
56-60 ..	20	27	32	9	49	35	37	5	214	31	7	..
61-65 ..	9	10	9	2	13	6	12	..	61	10	2	..
66-70 ..	3	7	7	2	7	11	10	5	52	3	1	..
71-75 ..	1	3	..	..	3	4	1	1	13	2	..	..
76-80 ..	..	3	0	2	2	2	1	..	10	..	..	..
81 and above ..	..	..	1	..	..	..	..	5	1	..	..	..
Unknown ..	33	24	17	13	50	37	55	..	233	51	10	2
TOTALS ..	429	222	284	113	403	284	412	24	2,171	552	129	36

TABLE VI.

*Showing distribution of malignant disease according to sex, religion, and site from pathological histology records availed of during the survey.*

Religion and sex.	CARCINOMA.								SARCOMA.			Teratoma.	Endothelioma.	Hodgkin's disease.
	Female genitals.	Penis.	Breast.	Gastro-intestinal tract and liver.	Skin.	Buccal.	Miscellaneous.	Rodent ulcer.	TOTAL.	Soft.	Hard.	TOTAL.		
Hindu males ..	0	171	9	61	128	189	177	1	736	191	74	265	45	17
Hindu females ..	263	0	152	17	51	69	66	2	620	111	18	129	39	2
Mohammedan males	0	1	0	4	11	41	23	3	83	21	10	31	5	1
Mohammedan females	18	0	14	0	9	10	13	1	65	6	2	8	4	1
Burmese males ..	..	31	3	6	18	26	40	4	128	18	6	24	7	7
Burmese females ..	81	..	56	3	12	17	24	1	194	25	5	30	5	2
Other males ..	0	15	1	8	19	36	34	7	120	15	7	22	7	5
Other females ..	49	0	42	7	11	9	10	3	131	12	5	17	9	0
Unknown ..	18	4	7	7	25	6	25	2	91	18	8	26	8	1
TOTALS ..	429	222	284	113	284	403	412	24	2,171	417	135	552	129	36

TABLE VII.

*Regional distribution of carcinoma per hundred cases based on the biopsy records of the pathology laboratories of the following institutions.*

Name of institution.	Female genitals.	Penis.	Breast.	Gastro-intestinal tract and liver.	Buccal.	Skin.	Miscellaneous.	Rodent ulcer.
Medical College, Rangoon ..	21.4	7.2	15.0	3.6	19.9	8.9	21.6	1.8
Medical College, Madras, and Raipurum Medical School, Madras.	19.2	11.3	12.3	5.7	18.0	14.5	17.8	0.8
Percentage incidence for the above institutions taken together.	19.7	10.2	13.0	5.2	18.5	13.0	18.9	1.1

TABLE VIII.

*Regional distribution of carcinoma per hundred male cases based on the records of the pathology laboratories of the following institutions.*

Name of institution.	Female genitals.	Penis.	Breast.	Gastro-intestinal tract and liver.	Buccal.	Skin.	Miscellaneous.	Rodent ulcer.	Total cases.
Medical College, Rangoon ..	..	14.8	1.8	5.0	30.5	11.8	32.8	3.2	279
Medical College, Madras, and Raipurum Medical School, Madras.	..	22.4	1.0	8.2	26.2	18.1	23.1	0.7	788
Percentage incidence for the above institutions taken together.	..	20.7	1.1	7.2	27.3	16.5	25.5	1.4	1,067

TABLE VIIIa.

*Regional distribution of carcinoma per hundred Hindu male cases based on the records of the pathology laboratories of the following institutions.*

Name of institution.	Penis.	Breast.	Gastro-intestinal tract and liver.	Buccal.	Skin.	Miscellaneous.	Rodent ulcer.	Total cases.
Medical College, Rangoon ..	11.8	2.7	5.2	39.5	9.2	31.5	..	76
Medical College, Madras, and Raimpurn Medical School, Madras.	24.5	1.06	8.6	24.1	17.9	23.2	0.15	660

TABLE VIIIb.

*Regional distribution of carcinoma per hundred Mohammedan male cases based on the records of the pathology laboratories of the following institutions.*

Name of institution.	Penis.	Breast.	Gastro-intestinal tract and liver.	Buccal.	Skin.	Miscellaneous.	Rodent ulcer.	Total cases.
Medical College, Rangoon ..	2.7	..	8.3	50.0	5.5	33.3	..	36
Medical College, Madras, and Raimpurn Medical School, Madras.	..	..	2.1	48.9	69.1	23.4	5.3	47

TABLE VIIIc.

*Regional distribution of carcinoma per hundred other male cases based on the records of histology laboratories of the following institutions.*

Name of institution.	Penis.	Breast.	Gastro-intestinal tract and liver.	Buccal.	Skin.	Miscellaneous.	Rodent ulcer.	Total cases.
Medical College, Rangoon ..	..	..	2.5	15.3	28.2	41.0	13.0	39
Medical College, Madras, and Rairupurum Medical School, Madras.	18.5	1.2	8.6	30.8	16.05	22.0	2.3	81

TABLE VIId.

*Regional distribution of carcinoma per hundred Burmese male cases based on the records of the pathology laboratories of the Medical College, Rangoon.*

Name of institution.	Penis.	Breast.	Gastro-intestinal tract and liver.	Buccal.	Skin.	Miscellaneous.	Rodent ulcer.	Total cases.
Medical College, Rangoon ..	24.2	2.4	4.7	20.3	14.0	31.2	3.1	128

TABLE IX.

*Regional distribution per hundred female cases based on the records of the pathology laboratories of the following institutions.*

Name of institution.	CANCER.						
	Female genitals.	Breast.	Gastro- intestinal tract and liver.	Buccal.	Skin.	Miscellaneous.	Rodent ulcer.
Medical College, Rangoon	44.7	27.8	2.4	9.9	5.8	12.0	0.7
Medical College, Madras, and Raimun Medical School, Madras.	40.4	25.4	2.7	10.5	9.1	10.8	0.7
Percentage incidence of the above institutions taken together.	40.6	27.1	2.6	10.3	8.2	11.1	0.69
							1,010

TABLE IXa.

*Regional distribution of carcinoma per hundred Hindu female cases based on the records of the pathology laboratories of the following institutions.*

Name of institution.	CANCER.						
	Female genitals.	Breast.	Gastro- intestinal tract and liver.	Buccal.	Skin.	Miscellaneous.	Rodent ulcer.
Medical College, Rangoon	47.2	13.9	..	13.9	8.3	16.6	..
Medical College, Madras, and Raimun Medical School, Madras.	42.1	25.1	2.9	10.9	8.5	10.3	0.3
							36
							584

TABLE IXb.

*Regional distribution of carcinoma per hundred Mohammedan female cases based on the histology records of the following institutions.*

Name of institution.	Female genitals.	Breast.	Gastro- intestinal tract and liver.	Buccal.	Skin.	Miscellaneous.	Rodent ulcer.	Total cases.
Medical College, Rangoon ..	33.3	16.6	..	25.0	8.3	16.6	..	12
Medical College, Madras, and Rairupurum Medical School, Madras.	26.7	22.6	..	13.2	15.1	20.7	1.9	53

TABLE IXc.

*Regional distribution of carcinoma per hundred other female cases based on the histology records of the following institutions.*

Name of institution.	Female genitals.	Breast.	Gastro- intestinal tract and liver.	Buccal.	Skin.	Miscellaneous.	Rodent ulcer.	Total cases.
Medical College, Rangoon ..	36.7	36.7	8.3	8.3	2.0	6.0	2.0	49
Medical College, Madras, and Rairupurum Medical School, Madras.	37.7	29.2	10.9	4.8	6.1	8.5	2.4	82

TABLE IXd.

*Regional distribution of carcinoma per hundred Burmese female cases based on the histology records of the Medical College, Rangoon.*

Name of institution.	Female genitals.	Breast.	Gastro- intestinal tract and liver.	Buccal.	Skin.	Miscellaneous.	Rodent ulcer.	Total cases.
Medical College, Rangoon ..	41.7	28.9	1.5	8.7	6.1	12.2	0.5	194

TABLE X.

*Neoplastic diseases as shown in the records of the following institutions.*

Number.	Name of institution.	Total number of in-patients treated.	Tera- toma.	Carci- noma.	Sarcoma.	Endo- thelioma.	Hodgkin's disease.	Ratio of sarcoma to car- cinoma.	Ratio of cases of malignant diseases to total number of in-patients treated.
1	General Hospital, Rangoon.	58,972	22	704	107	3	18	1: 7.0	1: 69
2	General Hospital, Madras.	74,827 (1930-34)	21	2,632	310	2	18	1: 8.4	1: 25
3	Maternity Hospital, Madras.	38,290 (1930-33)	1	1,375	5	..	..	1: 275.0	1: 28
4	Raipuram Medical School, Madras.	50,353 (1930-34)	6	472	71	..	9	1: 6.6	1: 89
Totals ..		222,442	50	5,183	493	5	45	1: 10.5	1: 38.5



TABLE XI.  
Incidence of malignant disease as based on hospital records of the following institutions.

Name of institution.	CANCER.								SARCOMA.			Teratoma.	Endothelioma.	Hodgkin's disease.
	Female genitals.	Penis.	Breast.	Gastro-intestinal tract and liver.	Buccal.	Skin.	Miscellaneous.	Rodent ulcer.	Total.	Soft.	Hard.	Total.		
General Hospital, Madras.	41	185	235	351	1,538	52	207	23	2,632	172	138	310	21	18
Raipurum Medical School, Madras.	43	69	60	64	159	28	43	6	472	46	25	71	6	9
Maternity Hospital, Madras.	1,308	0	27	17	..	4	19	..	1,375	4	1	5	1	..
General Hospital, Rangoon.	110	46	65	194	120	26	135	8	704	62	45	107	22	18
Lady Dufferin Hospital, Rangoon.	166	..	14	1	4	..	10	..	195	1	..	1	1	..
TOTALS ..	1,668	300	401	627	1,821	110	414	37	5,378	285	209	494	50	45

TABLE XII.

Showing distribution of carcinoma according to age and site and of sarcoma according to age alone as gathered from hospital records of the Medical Colleges, Rangoon and Madras, and the Medical School, Raipuram.

Age.	CARCINOMA.										Sarcoma.	Teratoma.	Endothelioma.	Hodgkin's disease.
	Female genitals.	Penis.	Breast.	Gastro-intestinal tract and liver.	Buccal.	Skin.	Miscellaneous.	Rodent ulcer.	TOTAL.					
1-5	..	..	1	..	2	..	2	..	5	20	1	..	3	
6-10	..	..	..	..	4	..	3	..	7	25	1	..	4	
11-15	..	..	1	..	5	1	4	..	12	23	2	..	0	
16-20	..	1	4	6	12	8	11	1	50	61	2	..	4	
21-25	..	7	10	15	44	5	15	2	147	57	2	..	4	
26-30	..	49	31	54	113	12	31	0	429	55	3	..	4	
31-35	..	165	30	49	179	9	36	2	569	52	6	..	4	
36-40	..	238	30	86	407	13	57	2	999	57	6	..	4	
41-45	..	333	61	79	244	7	45	2	726	32	1	..	6	
46-50	..	264	47	108	334	20	57	5	926	37	7	..	5	
51-55	..	265	84	67	173	7	40	6	532	16	6	..	3	
56-60	..	169	50	71	194	6	49	2	524	11	1	..	2	
61-65	..	101	45	33	77	7	14	2	196	9	1	..	..	
66-70	..	33	16	13	50	1	13	4	105	5	1	..	..	
71-75	..	13	4	4	14	3	8	2	41	1	..	..	..	
76-80	..	3	4	2	6	1	..	..	13	1	..	..	..	
80 and over	..	2	1	2	1	1	..	..	5	1	..	..	..	
Unknown	..	..	11	38	62	9	29	2	92	31	4	..	5	
TOTALS ..	1,668	300	401	627	1,821	110	414	37	5,378	494	50	6	45	

TABLE XIII.

Combined table showing cases of malignant disease according to sex, religion, and site in the Province of Burma and Madras Presidency.

Religion and sex.	CANCER.								SARCOMA.			Teratoma.	Endothelioma.	Hodgkin's disease.
	Female genitals.	Penis.	Breast.	Gastro-intestinal tract and liver.	Buccal.	Skin.	Miscellaneous.	Rodent ulcer.	TOTAL.	Hard.	Soft.	TOTAL.		
Hindu males ..	..	221	19	306	1,039	57	169	12	1,823	125	168	293	18	20
Hindu females ..	1,223	..	205	90	343	18	64	9	1,952	31	46	77	7	6
Mohammedan males	..	1	2	53	159	2	34	2	253	15	17	32	4	4
Mohammedan females	49	..	11	4	34	..	9	..	107	3	4	7	2	0
Burmese males ..	..	34	..	70	25	19	41	3	192	16	13	29	7	7
Burmese females ..	181	..	63	25	14	2	31	1	320	2	16	18	5	0
Other males ..	..	15	5	58	164	7	45	6	300	11	15	26	4	8
Other females ..	201	..	73	18	40	4	19	4	359	2	4	6	1	..
Unknown ..	11	29	23	3	3	1	2	..	72	4	2	6	2	0
TOTALS ..	1,668	300	401	627	1,821	110	414	37	5,378	209	285	494	50	45

TABLE XIV.

Combined table showing cases of malignant disease according to sex, religion, and site in the following institutions :—

(1) General Hospital, Madras, (2) Raipuram Medical School, Madras, and  
(3) Maternity Hospital, Madras.

Religion and sex.	CANCER.								SARCOMA.			Teratoma.	Endothelioma.	Hodgkin's disease.
	Female genitals.	Penis.	Breast.	Gastro-intestinal tract and liver.	Buccal.	Skin.	Miscellaneous.	Rodent ulcer.	TOTAL.	Hard.	Soft.	TOTAL.		
Hindu males ..	0	212	19	269	997	53	143	12	1,705	110	153	263	16	17
Hindu females ..	1,175	0	202	85	338	17	58	9	1,884	28	42	70	7	5
Mohammedan males	0	1	2	31	138	2	21	2	197	13	10	23	2	3
Mohammedan females	37	0	11	3	27	0	6	0	84	2	2	4	1	0
Other males ..	0	12	4	32	154	7	25	3	237	8	10	18	1	2
Other females ..	173	0	61	10	40	4	14	3	305	1	3	4	0	0
Unknown ..	7	29	23	2	3	1	2	0	67	2	1	3	1	0
TOTALS ..	1,392	254	322	432	1,697	84	269	29	4,479	164	221	385	28	27

TABLE XIVa.

Combined table showing cases of malignant disease according to sex, religion, and site in the following institutions :—

(1) General Hospital, Rangoon, and (2) Lady Dufferin Hospital, Rangoon.

Religion and sex.	CANCER.							SARCOMA.				Teratoma.	Endothelioma.	Hodgkin's disease.	
	Female genitals.	Penis.	Breast.	Gastro-intestinal tract and liver.	Buccal.	Skin.	Miscellaneous.	Rodent ulcer.	Total.	Soft.	Hard.				Total.
Hindu males ..	0	9	0	37	42	4	26	0	118	15	15	30	2	1	3
Hindu females ..	48	0	3	5	5	1	6	0	68	3	4	7	0	0	1
Mohammedan males	0	0	0	22	21	1	13	0	56	7	2	9	2	0	1
Mohammedan females	12	0	0	1	7	0	3	0	23	2	1	3	1	0	0
Burmese males ..	0	34	0	70	25	19	41	3	192	13	16	29	7	0	7
Burmese females ..	184	0	63	25	14	2	31	1	320	16	2	18	5	2	0
Other males ..	0	3	1	26	10	0	20	3	63	5	3	8	3	1	6
Other females ..	28	0	12	8	0	0	5	1	54	1	1	2	1	0	0
Unknown ..	4	0	0	1	0	0	0	0	5	0	2	2	1	0	0
Totals ..	276	46	89	195	124	26	145	8	899	63	46	108	22	4	18

TABLE XV.

*Incidence of neoplastic diseases according to provinces as ascertained from the records of hospitals visited.*

Name of province.	CANCER.								SARCOMA.				Endothelioma.	Hodgkin's disease.
	Female genitals.	Penis.	Breast.	Gastro-intestinal tract and liver.	Buccal.	Skin.	Miscellaneous.	Rodent ulcer.	TOTAL.	Hard.	Soft.	TOTAL.		
Madras ..	1,392	254	322	432	1,697	84	269	29	4,479	164	222	386	27	
Burma ..	276	46	79	195	124	26	145	8	899	45	63	108	18	

TABLE XVI.

*Regional distribution per hundred male cases according to communities in the Madras Presidency.*

Name of community.	Penis.	Breast.	Gastro-intestinal tract and liver.	Buccal.	Skin.	Miscellaneous.	Rodent ulcer.	TOTAL.	Actual number of cases recorded.
Hindu ..	12.5	1.1	15.7	58.5	3.1	8.4	0.7	100	1,705
Mohammedan ..	0.5	1.0	15.6	70.0	1.0	10.5	1.0	100	197
Others ..	5.1	1.7	13.1	65.0	2.95	10.5	1.3	100	237
For 100 male cases ..	10.55	1.1	15.55	60.15	2.95	8.8	0.8	100	2,139
For combined Burma and Madras.	10.5	0.9	18.9	54.0	3.3	..	..	..	..

TABLE XVIa.  
*Regional distribution of carcinoma per hundred females according to communities in the Madras Presidency.*

Name of community.	Genitals.	Breast.	Gastro-intestinal tract and liver.	Buccal.	Skin.	Miscellaneous.	Rodent ulcer.	Total.	Actual number of cases recorded.
Hindu ..	62.3	10.7	4.5	17.9	0.9	3.0	0.45	100	1,884
Mohammedan ..	44.0	13.1	3.5	32.1	..	7.1	0	100	84
Others ..	56.7	20.0	3.2	12.9	1.3	4.5	0	100	305
For 100 female cases	60.9	12.05	4.3	17.9	0.9	3.8	0.5	100	2,273
For combined Burma and Madras.	60.5	12.8	5.0	15.3	0.8	..	..	..	..

TABLE XVIb.  
*Regional distribution of carcinoma per hundred males according to communities in the Province of Burma.*

Name of community.	Penis.	Breast.	Gastro-intestinal tract and liver.	Buccal.	Skin.	Miscellaneous.	Rodent ulcer.	Total.	Actual number of cases recorded.
Hindu ..	7.6	..	31.3	35.5	3.6	22.0	..	100	118
Mohammedan ..	..	..	39.1	37.8	..	23.1	..	100	56
Burmese ..	17.7	..	36.5	13.0	9.9	21.3	1.6	100	192
Others ..	4.8	1.6	41.3	15.6	..	31.3	4.8	..	63
For 100 male cases	10.8	0.3	36.1	22.9	5.4	23.3	1.4	100	429
For combined Burma and Madras.	10.5	0.9	18.9	54.0	3.3	..	..	..	..

TABLE XVIc.

*Regional distribution of carcinoma per hundred female cases according to communities in the Province of Burma.*

Name of community.	Female genitals.	Breast.	Gastro- intestinal tract and liver.	Buccal.	Skin.	Miscellan- eous.	Rodent ulcer.	TOTAL.	Actual number of cases recorded.
Hindu ..	70.6	4.4	7.3	7.3	1.4	8.5	..	100	68
Mohammedan ..	52.1	..	4.3	30.4	..	13.0	..	100	23
Burmese ..	57.5	19.7	7.8	4.4	0.6	9.7	0.3	100	320
Others ..	51.8	22.2	14.8	..	..	9.2	1.8	100	54
For 100 female cases ..	58.5	16.9	8.45	5.6	0.7	8.5	0.4	100	465
For combined Burma and Madras.	60.5	12.8	5.0	15.3	0.8	..	..	..	..

TABLE XVII.

*Regional distribution of carcinoma per hundred cases according to provinces as ascertained from the records of hospitals visited.*

Name of province.	Female genitals.	Penis.	Breast.	Gastro- intestinal tract and liver.	Buccal.	Skin.	Miscellan- eous.	Rodent ulcer.	TOTAL.	Actual number of cases recorded.
Madras ..	31.08	5.45	7.25	9.65	37.9	1.9	6.0	0.64	100	4,479
Burma ..	30.7	5.1	8.8	21.7	13.9	2.9	16.1	0.89	100	899



TABLE XVIIa.

*Regional distribution of carcinoma per hundred male cases according to provinces as ascertained from the records of hospitals visited.*

Name of province.	Penis.	Breast.	Gastro-intestinal tract and liver.	Buccal.	Skin.	Miscellaneous.	Rodent ulcer.	TOTAL.	Actual number of cases recorded.
Madras ..	10.55	1.1	15.55	60.15	2.95	8.8	0.8	100	2,139
Burma ..	10.8	0.3	36.1	22.9	5.4	23.3	1.4	100	429

TABLE XVIIb.

*Regional distribution of carcinoma per hundred Hindu male cases according to provinces as ascertained from the records of hospitals visited.*

Name of province.	Penis.	Breast.	Gastro-intestinal tract and liver.	Buccal.	Skin.	Miscellaneous.	Rodent ulcer.	TOTAL.	Actual number of cases recorded.
Madras ..	12.5	1.1	15.7	58.5	3.1	8.4	0.7	100	1,705
Burma ..	7.6	..	31.3	35.5	3.6	22.0	..	100	118

TABLE XVIIc.

*Regional distribution of carcinoma per hundred Mohammedan male cases according to provinces.*

Name of province.	Penis.	Breast.	Gastro-intestinal tract and liver.	Buccal.	Skin.	Miscellaneous.	Rodent ulcer.	TOTAL.	Actual number of cases recorded.
Madras ..	0.5	1.0	15.6	70.0	1.0	10.5	1.0	100	197
Burma ..	..	..	30.1	37.8	..	23.1	..	100	56

TABLE XVIIId.

*Regional distribution of carcinoma per hundred Burmese male cases.*

Name of province.	Penis.	Breast.	Gastro-intestinal tract and liver.	Buccal.	Skin.	Miscellaneous.	Rodent ulcer.	TOTAL.	Actual number of cases recorded.
Burma ..	17.7	..	36.5	13.0	9.9	21.3	1.6	100	192

TABLE XVII.

*Regional distribution of carcinoma per hundred other male cases according to provinces.*

Name of province.	Penis.	Breast.	Gastro-intestinal tract and liver.	Buccal.	Skin.	Miscellaneous.	Rodent ulcer.	TOTAL.	Actual number of cases recorded.
Madras ..	5.1	1.7	13.1	65.0	2.95	10.5	1.3	100	237
Burma ..	4.8	1.6	41.3	15.6	0	31.3	4.8	..	63

TABLE XVIII.

*Regional distribution of carcinoma per hundred female cases according to provinces as ascertained from the records of hospitals visited.*

Name of province.	Female genitals.	Breast.	Gastro-intestinal tract and liver.	Buccal.	Skin.	Miscellaneous.	Rodent ulcer.	TOTAL.	Actual number of cases recorded.
Madras ..	60.9	12.05	4.3	17.9	0.9	3.8	0.5	100	2,273
Burma ..	58.5	16.9	8.45	5.6	0.7	8.5	0.4	100	465

TABLE XVIIIa.

*Regional distribution of carcinoma per hundred Hindu female cases according to provinces as ascertained from the records of hospitals visited.*

Name of province.	Female genitals.	Breast.	Gastro- intestinal tract and liver.	Buccal.	Skin.	Miscellan- eous.	Rodent ulcer.	TOTAL.	Actual number of cases recorded.
Madras ..	62.3	10.7	4.5	17.9	0.9	3.0	1.45	100	1,884
Burma ..	70.6	4.4	7.3	7.3	1.4	8.5	0	100	68

TABLE XVIIIb.

*Regional distribution of carcinoma per hundred Mohammedan female cases according to provinces.*

Name of province.	Female genitals.	Breast.	Gastro- intestinal tract and liver.	Buccal.	Skin.	Miscellan- eous.	Rodent ulcer.	TOTAL.	Actual number of cases recorded.
Madras ..	44.0	13.1	3.5	32.1	..	7.1	..	100	84
Burma ..	52.1	..	4.3	30.4	0	13.0	..	100	23

TABLE XVIIIc.

*Regional distribution of carcinoma per hundred Burmese female cases.*

Name of province.	Female genitals.	Breast.	Gastro- intestinal tract and liver.	Buccal.	Skin.	Miscellan- eous.	Rodent ulcer.	TOTAL.	Actual number of cases recorded.
Burma ..	57.5	19.7	7.8	4.4	0.6	9.7	0.3	100	320

TABLE XVIIIId.

*Regional distribution of carcinoma per hundred other female cases according to provinces.*

Name of province	Female genitals.	Breast.	Gastro- intestinal tract and liver.	Buccal.	Skin.	Miscellan- eous.	Rodent ulcer.	TOTAL.	Actual number of cases recorded.
Madras ..	56.7	20.0	3.2	12.9	1.3	4.5	0.9	100	305
Burma ..	51.8	22.2	14.8	..	..	9.2	1.8	100	54

TABLE XIX.

*Incidence of cancer as ascertained from the records of the Inspector-General of Civil Hospitals, Burma, and from the records of the Surgeon-General, Madras.*

Name of province.	Period.	Total number of in-patients treated in Class I, III and IV hospitals of the province.	Total number of cases of malignant disease treated as in-patients.	Ratio of cases of malignant disease to total number of in-patients.
Burma .. ..	1930-33 both inclusive.	432,446	2,631	1 : 164
Madras .. ..	1930-33	851,029	9,558	1 : 89

## DIET SURVEYS IN SOUTH INDIAN VILLAGES.

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It is often said that the diet of the Indian villager or ryot is insufficient in quantity and poor in quality. On the other hand, statements are made that the average villager, in spite of his manifest poverty, has enough 'good wholesome food' to maintain health and strength, and that the diet of the poor in towns and cities is much more deficient. The general impressions of untrained observers on such points are, however, of doubtful value, and very little exact information, collected by systematic surveys, is available about the diet of Indian peasants. Since some 80 per cent of the population live in villages, such information is essential if the problem of nutrition in India is to be defined and understood, and its collection is one of the most important objects of nutrition research.

McCay (1912) gives the composition of 'average diets' consumed by various classes of the population in Bengal, including cultivators, and also that of an 'average' Sikh diet. The dietaries of industrial workers in India have been investigated in family budget inquiries carried out by the Labour Office of the Government of Bombay (1923, 1928*a* and *b*, 1935) and by the Commerce Department of the Government of Bengal (1930); the purpose of these investigations was, however, economic, and no attempt was made to assess the physiological adequacy of the diets. Wilson, Ahmad and Mullick (1936) have recently made a detailed study of the diet of a few families in Calcutta. The Board of Economic Inquiry, Punjab (1928), has published an economic survey of a village in the Amritsar district in which some rough data about the food intake of a few families are included; it is possible that the reports of economic inquiries carried out elsewhere in the country contain similar data. Nicholls (1936) has reported the results of an investigation of the diet of 16 Ceylonese families of the labouring classes, and a similar but more detailed inquiry, extending over a year and including 15 families of peasants and 5 families of working men, has been carried out in Java (Ochese *et al.*, 1934). Surveys in adjacent countries where dietary habits are roughly similar are of interest to nutrition workers in India.

## SCOPE AND METHOD OF THE PRESENT INQUIRY.

The diet of 44 families, including 274 persons, was investigated over a period of 20 days. Of these, 8 were Christian families, engaged in cultivation and coolie work, living in a small village near Chingleput in South-East Madras. Four Hindu families, distinctly more prosperous, living in a neighbouring village, were included in this survey which took place in January 1936. A larger inquiry, comprising 32 families, was organized in a number of small villages in the neighbourhood of Mayanur, about 36 miles N.-W. of Trichinopoly; this inquiry took place in July and August 1936. Twenty-nine of these were engaged in agriculture or ancillary village occupations, with some coolie work of various kinds. The occupations of the remainder were as follows: clerk in the Public Works Department; agricultural instructor at a Rural Reconstruction Centre; owner of a provision store.

The actual collection of the data was carried out, in the case of the Chingleput group, by student health visitors of the Red Cross School, Madras, and a village nurse, working under the supervision of a European lady missionary. In the Mayanur inquiry, 6 teachers attached to the Servants of India Society Rural Reconstruction Centre at Mayanur collaborated. To carry out such inquiries successfully it is essential to work in co-operation with agents on familiar terms with the families under investigation and trusted by them. At the outset we carefully explained to our collaborators what we wanted them to do, and remained with them for a few days of supervision, during which homes were visited, food weighed out, and the details filled in on an appropriate form. When we were sure that they had fully grasped the method and purpose of the inquiry, we left them to 'carry on' themselves. They visited each home twice daily at the time of the preparation of meals and weighed each item of food to be consumed on portable balances. In the Mayanur inquiry each worker visited 6 families daily; apart from the fact that the teachers concerned had other work to do, this is about the maximum number that one worker can deal with at a time, because families must be visited early at the time of preparing the food for the day. The question of making a conventional allowance for 'waste', as is usual in family dietary inquiries, scarcely arises. If food prepared on one day was not entirely consumed, it would be kept for the next and added to that day's reckoning. It is always advisable, in presenting the results of dietary inquiries, to describe the actual method of field investigation; when such information is not given, one has no means of assessing the reliability of the final conclusions. Our experience in field dietary inquiries may be of value to other investigators attempting this line of research. We have little doubt of the substantial accuracy of the food-intake data collected in these inquiries. To the best of our knowledge, these are the first detailed village-diet surveys to be carried out in India.

It may be objected that a three-weeks' inquiry is inadequate to give a picture of diet throughout the year, and that for a complete survey repeated investigations at other seasons would be desirable. In actual fact, however, the diet of very poor South Indian peasants does not vary much with season and the results of even a short survey at one season may throw light on food intake throughout the year. In the Chingleput area, the chief rainy season is in October, November,



and December, so that January follows the rains ; in the Mayanur district there had been fair rain during the month previous to the inquiry.

In the Mayanur district, 323 school children belonging to the families studied and other families of similar status were weighed and measured and examined for evidence of food deficiency disease. No examination of adults was carried out.

To show the attitude of the families to the investigation, we cannot do better than quote a passage from a letter from Mr. A. L. Subramanyan who participated actively in the Mayanur inquiry :—

‘ In the case of some families there was no difficulty even from the beginning. It was necessary to create confidence by removing their prejudice, suspicion, fear, and general apathy, all due to their poverty and illiteracy. Only the 32 families for which we are able to send figures co-operated with us to the very end. About half a dozen dropped off after a few days. In some families the male members were favourably inclined though the women protested ; in others, it was the reverse. The prejudice against weighing things to be cooked and eaten was hard to break. “ How is this weighing of our food grains going to benefit us ? Will it solve our poverty problem ? Will it help us to feed our hungry mouths ? ” were the usual remarks. Their poverty and misery, which we saw at close quarters, you may be able to see from the figures we are submitting ’.

#### WORKING UP OF THE DATA.

Intake of calories, proximate principles, calcium, phosphorus, and iron was calculated per consumption unit per day. To reduce families of varying age and sex composition to consumption units, the earlier International scale of family co-efficients (League of Nations Health Organization, 1932) was employed. This scale is as follows :—

Age.	CO-EFFICIENT.		
	Male.	Both sexes.	Female.
0-2 ..	..	0.2	..
2 and 3 ..	..	0.3	..
4 „ 5 ..	..	0.4	..
6 „ 7 ..	..	0.5	..
8 „ 9 ..	..	0.6	..
10 „ 11 ..	..	0.7	..
12 „ 13 ..	..	0.8	..
14 to 59 ..	1.0	..	0.8
Over 60 ..	..	0.8	.

It must be emphasized that at present we have no knowledge regarding the appropriateness of this scale for use in India. It has been employed by Nicholls (*loc. cit.*) for a similar inquiry in Ceylon, and there is no reason to suppose that any other scale in current use (e.g., Lusk's or Cathcart and Murray's) would be more satisfactory. Only by systematic investigation of the basal metabolism and food intake of both sexes at various ages can a suitable Indian scale of family consumption co-efficients be constructed.

In calculating intake of the various food factors, figures obtained in a systematic survey of the composition of Indian foods, carried out in the laboratory, were used (Ranganathan *et al.*, 1937). Analyses were made on the edible portion of the raw uncooked food.

Families have been divided into 4 groups as follows :—

GROUP I. Eight families in Overtownpet village, near Chingleput. Sixty-seven persons.

GROUP II. Four families in Karumpakkum village, near Chingleput. Twenty-eight persons.

GROUP III. Twenty-nine agricultural families in various villages in the neighbourhood of Mayanur, Trichinopoly district. One hundred and sixty-eight persons.

GROUP IV. Three families not engaged in agriculture in same district. Eleven persons.

#### ECONOMIC STATUS.

To arrive at a satisfactory assessment of the economic position of any group of peasant families, it would be necessary to collect data about all sources of income, indebtedness, taxes, the amount of live stock and other property owned, etc. Indian village economy is complicated, and its detailed study lies outside the scope of nutrition research. An attempt was nevertheless made, in the present investigation, to assess gross income very roughly, in terms of the value of crops produced and wages obtained for coolie work and other labour.

GROUP I was the poorest of the four. The families in this group were tenants with an average holding of 3 acres of land per household, for which rent was paid in cash and produce to the value of Rs. 3 per acre per annum. Average annual income in this group may be roughly reckoned as between Rs. 50 and Rs. 80 per family (with the exception of one more prosperous family).

GROUP II consisted of families of a distinctly more prosperous order. The total income of family 1 in this group, derived from the father's occupation as school teacher and postmaster as well as from agriculture, exceeded Rs. 1,000 per annum. In the other 3 families, annual income lay between Rs. 200 and Rs. 300.

GROUP III included families of different income and economic status, and may perhaps be regarded as a cross section of a village community. At the upper limit, we may place a Brahmin family owning 10 acres of wet land, and a considerable quantity of live stock, whose annual income may be reckoned as Rs. 300; at the lower, families supported by coolie work, leasing an acre or so of dry land, and owning one or two head of cattle, with a gross annual income under Rs. 100. Average family income in this group was somewhat higher than in Group I, being in the neighbourhood of Rs. 100 per annum. The incomes of Group IV lay between Rs. 350 and Rs. 500.

Most of the families owned some live stock including cows and she-buffaloes. During the period of investigation, however, the out-turn of milk was very small, and a number of families sold a high proportion of whatever milk was produced. Only one or two families owned sheep and goats; goat's milk was not consumed at all. Poultry were unimportant as a source of income or food.

TABLE I.

*Intake of calories, proximate principles, calcium, phosphorus, and iron per consumption unit per day.*

Family.	Number of persons.	Number of consumption units.	Protein (g.).	Fat (g.).	Carbo-hydrates (g.).	Calories.	Percentage of total calories derived from cereals.	Calcium (g.).	Phosphorus (g.).	Iron (mg.).
1	14	9.9	44.1	5.3	447.9	2,026	96	0.52	0.90	23.5
2	6	4.8	40.9	6.4	414.5	1,896	95	1.18	0.93	30.1
3	6	4.8	37.6	4.9	392.8	1,775	94	0.10	0.74	17.8
4	11	7.7	34.0	3.6	387.0	1,725	98	0.05	0.69	14.8
5	9	6.1	37.6	4.0	358.2	1,601	96	0.98	0.93	28.5
6	7	4.4	32.5	3.6	351.2	1,576	96	0.45	0.69	19.0
7	6	3.9	31.7	3.2	341.8	1,529	96	1.19	0.72	32.1
8	8	5.1	28.7	3.6	257.7	1,184	92	0.33	0.57	13.8
TOTAL	67	Mean 5.8	35.9	4.4	368.9	1,664	95.4	0.60	0.75	22.4
1	9	7.6	52.8	37.9	478.3	2,473	77	0.72	1.13	23.8
2	7	6.4	50.9	14.9	483.2	2,280	88	0.48	1.02	24.7
3	7	5.6	44.1	10.2	429.6	1,995	89	0.39	0.90	21.7
4	5	3.2	44.2	19.9	394.5	1,946	77	0.33	0.85	25.5
TOTAL	28	Mean 5.7	48.0	20.7	446.4	2,173	83	0.48	0.97	23.9

GROUP I.

GROUP II.

TABLE II.  
Composition of the diet of various families. (Oz. per consumption unit per day).

GROUP I.			GROUP II.	
Family 1.	Family 7.	Family 3.		
Home-pounded parboiled rice .. 17.40	Home-pounded parboiled rice .. 14.90	Home-pounded parboiled rice .. 15.20		
Ragi ( <i>Eleusine coracana</i> ) .. 2.10	Bengal gram .. 0.94	Dhal arhar .. 1.40		
Dhal arhar ( <i>Cajanus indicus</i> ) .. 0.09	Beans .. 0.14	Black gram ( <i>Phaseolus mungo</i> ) .. 0.10		
Bengal gram ( <i>Cicer arietinum</i> ) .. 0.03	Tamarind .. 0.46	Bengal gram .. 0.15		
Beans .. 0.20	Coriander seeds .. 0.08	Beans .. 0.33		
Tamarind .. 0.45	Onions .. 0.03	Tamarind .. 0.90		
Coriander seeds .. 0.05	Radish .. 0.16	Coriander seeds .. 0.16		
Onions .. 0.07	Green leafy vegetables .. 0.27	Sundakai ( <i>Solanum torvum</i> ) .. 0.09		
Gingelly oil .. 0.01	Crabs .. 2.80	Onions .. 0.33		
Crabs .. 0.50	Fish .. 0.13	Radish .. 0.40		
Fish .. 0.50		Sorakai ( <i>Lagenaria vulgaris</i> ) .. 0.75		
Mutton .. 0.20		Green leafy vegetables .. 0.50		
		Brinjal ( <i>Solanum melongena</i> ) .. 0.09		
		Drumstick ( <i>Moringa oleifera</i> ) .. .		
		Coco-nut .. 0.12		
		Gingelly oil .. 0.42		
		Fish .. 0.03		
		Whole milk .. 1.10		
		Curds .. 0.48		
		Butter-milk .. 0.12		
		Sugar .. 0.09		
		Sago .. .		

TABLE II—*contd.*  
*Composition of the diet of various families. (Oz. per consumption unit per day).*

## GROUP III.

Family 3.	Family 7.	Family 14.	Family 22.	Family 29.	
Roughly milled.. 14.80 parboiled rice.	1.70	Home-pounded parboiled rice.	3.00	Roughly milled parboiled rice.	8.28
Cholam ( <i>Sorghum andropogon</i> ).	Cholam .. 24.90	Cholam .. 17.40	Cholam .. 14.00	Cholam .. 2.79	
Dhal arhar .. 2.10	Dhal arhar .. 0.63	Camhu ( <i>Pennis- tum typhoidum</i> )	1.20	Ragi .. 2.10	Dhal arhar .. 0.95
Cluster beans .. 0.20	Tamarind .. 0.32	Dhal arhar .. 1.00	Dhal arhar .. 0.85	Tamarind .. 10.20	
Tamarind .. 1.10	Sweet potato 0.10	Black gram .. 0.32	Tamarind .. 0.40	Brinjal .. 0.75	
Amaranth leaves 0.80 ( <i>Amaranthus gan- geticus</i> ).	Brinjal .. 0.35	Tamarind .. 0.46	Amaranth leaves 0.41		
Brinjal .. 3.20	Chicken .. 0.37	Coriander seeds .. 0.02			
Coco-nut .. 0.15		Onions .. 0.17			
Gingelly oil .. 0.50		Amaranth leaves 0.70			
Fish .. 0.03		Brinjal .. 0.10			
Chicken .. 0.80		Plantains .. 1.40			
Jaggery .. 0.60		Gingelly oil .. 0.36			

TABLE II—concl'd.

Composition of the diet of various families. (Oz. per consumption unit per day).

GROUP IV.							
Provision merchant.							
Raw, milled rice	..	11.31	Brinjal	..	0.45	Jack-nuts ( <i>Artocarpus integrifolia</i> )	0.17
Parboiled rice	..	2.56	Pumpkin	..	0.30	Coco-nut ..	0.57
Dhal arhar ..	..	0.83	Lady's fingers ( <i>Vithiscus esculentus</i> )	..	0.22	Gingelly oil	0.85
Black gram ..	..	0.52	Snake gourd ( <i>Trichosanthus anguina</i> )	..	0.21	Gingelly seeds	0.10
Bengal gram ..	..	0.28	Potato	..	0.21	Whole milk	9.50
Mung dhal ( <i>Phaseolus radiatus</i> )	..	0.05	Drumstick ..	..	0.13	Curds ..	0.09
Tamarind ..	..	0.50	Drumstick leaves	..	0.09	Chee ..	0.90
Coriander seeds	..	0.15	Plantain (fruit)	..	0.27	Jaggery ..	0.35
Onions ..	..	0.06	Plantain (stem)	..	0.12	White sugar	0.90
			Mangoes ..	..	0.16		

## ANALYSIS OF DIETS.

Table I shows intake of calories, proximate principles and minerals, and the percentage of total calories, derived from cereals. Families are arranged in descending order of calorie intake.

In Table II, the composition of the diet of a number of families, as regards actual foodstuffs, is given. The families are chosen more or less at random out of the 4 groups as follows: Group I, families 1 and 7. Group II, family 3. Group III, families 3, 7, 14, 22, and 29. Group IV, provision merchant.

The average number of foods entering into the diets of the various groups during the period of investigation was as follows:—

GROUP I: 12 (range 8 to 18).

GROUP II: 20 ( „ 18 „ 24).

GROUP III: 11 ( „ 4 „ 24).

In Group IV the number of foods in the 3 diets was 33, 26 and 28 respectively. It is to be observed that many of the foods listed in the various diets shown in Table II were consumed only once or twice during the 20 days of the investigation.

Except in the case of a few prosperous families, foods other than cereals were consumed in very small quantities, as is apparent from Table II. In Group I the diet was composed almost exclusively of home-pounded parboiled rice; four of the group consumed a little ragi (millet). In Group II also, home-pounded parboiled rice formed a large proportion of the diet, but other foods were more abundantly present than in the diet of Group I. Group III ate both roughly milled parboiled rice and millet (usually cholam, less commonly cambu), the cereals in most instances providing 80 to 90 per cent of total calories.

*Milk.*—Milk products were absent from the diet of 31 out of the 44 families during the period of investigation. The remainder, including all the more prosperous families, consumed milk products in the form of milk, butter-milk, curds, or ghee. Quantities were as follows:—

GROUP II.

Family.	Product.	Quantity per consump- tion unit per day (oz.).
1	Whole milk ..	3.10
2	Curds ..	0.13
3	Whole milk ..	0.13
4	Whole milk ..	0.42
	Curds ..	1.10
	Butter-milk ..	0.48

## GROUP III.

Family.	Product.	Quantity per consump- tion unit per day (oz.).
8	Butter-milk ..	5.25
9	Butter-milk ..	0.16
11	{ Whole milk ..	3.72
	{ Curds ..	2.38
13	{ Whole milk ..	14.00
	{ Curds ..	3.50
	{ Ghee ..	0.68
17	Whole milk ..	0.34
26	Whole milk ..	2.17

## GROUP IV.

1	{ Whole milk ..	1.80
	{ Curds ..	3.10
2	{ Whole milk ..	1.35
	{ Curds ..	5.40
	{ Ghee ..	0.50
3	{ Whole milk ..	9.50
	{ Curds ..	0.09
	{ Ghee ..	0.90

It will be observed that only 3 families out of the whole group consumed ghee. The term 'curds' is applied to curdled milk, and the nutritive value of curds may be taken as equal to that of a similar quantity of whole milk.

*Animal foods other than milk.*—Only 3 out of the 44 families abstain from flesh foods because of religious conviction. The remainder eat mutton, fowl, fish, eggs, etc., when they can get these.

The majority of the families in Group I, during the short period of investigation, were eating small crabs caught in the local 'tanks' (irrigation ponds which fill up after the rains and dry up slowly during the non-rainy season). The supply of crabs lasts only a short time and this type of food is an unimportant part of the diet. One family during the period of investigation had a single meal which included a little chicken, and another including mutton. The families in this group consumed



also very small quantities of fish, averaging 0.25 oz. per day. Three of the families in Group II consumed fish in roughly similar quantities.

In Group III the diet of 10 families contained almost negligible amounts of fish, mutton, or chicken, the highest intake of foods of this nature being 0.80 oz. per c. u. daily. One family consumed a few eggs. No animal food apart from milk was included in the diet of Group IV.

Taking into consideration the small intake of milk and the virtual absence of meat and fish from the diet, it is obvious that the figures of protein intake given in Table II represent, for practical purposes, protein of vegetable origin.

*Pulses.*—The absence of animal protein from the diet, and the large proportional intake of cereals, make pulses an important ingredient in the diet of South Indian villagers. All the families under investigation consumed pulses, though some families, notably those in Group I, consumed only very small quantities. The most common pulse was dhal arhar (*Cajanus indicus*). The following indicates average consumption of pulses in the various groups :—

Mean

GROUP I : 0.26 oz. (range 0.08 to 0.36).

GROUP II : 1.29 „ ( „ 0.91 „ 1.98).

GROUP III : 1.32 „ ( „ 0.12 „ 3.97).

GROUP IV : 2.33 „ ( „ 1.30 „ 3.32).

*Vegetables.*—The Chinese are said to consume green leafy vegetables in large quantities, which results in the partial 'balancing' of their otherwise deficient diet. The same cannot be said of the South Indian families studied in the present investigation, at any rate during the period of inquiry. Only 4 of the families in Group I consumed green leafy vegetables, the highest consumption being 0.27 oz. per c. u. per day. In Group II consumption in 3 families averaged 0.30 oz., the other family consuming none. In Group III, 16 out of 31 families made use of leafy vegetables, average consumption among these being 0.40 oz. Consumption was low in Group IV (average of 3 families : 0.23 oz.).

Of non-leafy vegetables, brinjal (egg-plant) was the most commonly eaten. In Groups II, III, and IV it was absent from the diet of only 4 families ; Group I did not consume it at the season of inquiry. Other non-leafy vegetables eaten included drumstick, ridge gourd (*Luffa acutangula*), snake gourd, radish, onions, colacasia, and lady's fingers.

Omitting 4 families in Group III which did not use vegetables of this nature, average consumption in the various groups was as follows :—

GROUP I : 0.40 oz.

GROUP II : 2.70 „

GROUP III : 1.30 „

GROUP IV : 2.30 „

With regard to Group I, the missionary who kindly helped with the inquiry wrote a few weeks later as follows : ' You will notice the shortness of vegetables in the list. Since the recent rains the people have added some more vegetables to their diet '.

*Fruit.*—Fresh fruit was included in the diet of 17 families, the fruit most commonly consumed being plantain, green or ripe. The average intake of fruit in these families was 0·48 oz. per c. u. per day. The more prosperous families in Group IV consumed, in addition to plantains, mangoes in small quantities.

Tamarind (the dried fruit of the tree which commonly grows by the roadside in India) was present in the diet of all families, average intake being 0·55 oz. The consumption of the majority of families lay close to this figure. It would appear that a certain quantity of tamarind is included in the diet of even the poorest Indian peasant.

*Vegetable oils.*—Twenty-eight families consumed vegetable oil (gingelly), the average intake being 0·62 oz. Coco-nut, present in the diet of a few families, is included in this figure, having a high fat content. The diet of the remainder contained no vegetable oil or coco-nut.

#### EXAMINATION OF SCHOOL CHILDREN.

In order roughly to assess the state of nutrition associated with the dietary conditions observed in the Mayanur district, 323 children (boys), of ages ranging from 4 to 13, were examined in local schools. Some of these were drawn from the families of Group III; the remainder belonged to families consuming a similar kind of diet. It seems reasonable, in attempting to relate 'state of nutrition', and the results of village diet surveys in sample families, to examine a group of children in the area covered, irrespective of whether they all belong to the families included in the survey.

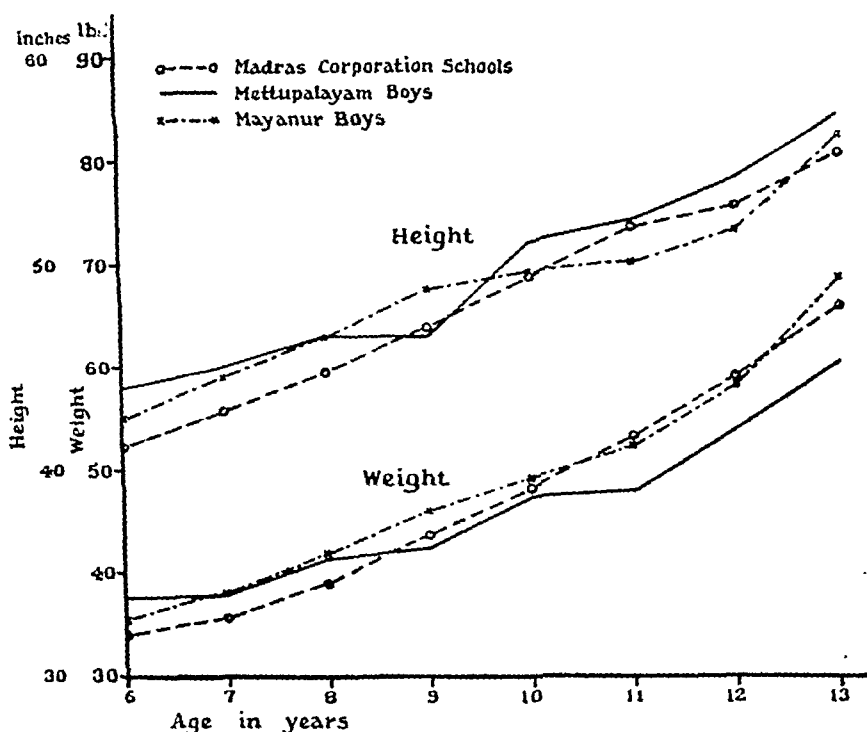
Heights and weights were taken, and the incidence of certain symptoms of food deficiency disease—phrynoderma, angular stomatitis, and Bitot's spots (xerophthalmia)—was observed. The method of clinical examination was that followed by Aykroyd and Rajagopal (1936) in carrying out surveys in South Indian towns.

Height and weight averages in the age groups 6 to 13 were as follows :—

Age.	Number of boys.	Height (inches).	Weight (lb.).
6	33	42·5	35·5
7	40	44·6	37·9
8	45	46·6	41·9
9	53	48·8	46·0
10	49	49·5	49·1
11	35	50·0	52·4
12	33	51·5	59·3
13	22	55·9	68·5

In Chart 1, these height and weight averages are compared with those of two other groups (boys): Madras Corporation schools (1934-1935), a very poor urban group in which a high incidence of malnutrition is reported; a semi-urban group in a small South Indian town, Mettupalayam (1936). It will be observed that the Mayanur averages correspond very closely with the Madras averages. In the Mettupalayam group, height averages are somewhat larger in the older age groups, and weight averages somewhat smaller. In a previous paper (Aykroyd and

CHART 1.



Rajagopal, *loc. cit.*) height and weight averages of South Indian boys were compared with those of boys in other countries, and it was suggested that the low 'weight for height' of Indian children, which is shown by the Mayanur group, is partly due to malnutrition.

In Table III, the percentage incidence of phrynoderma, Bitot's spots, and angular stomatitis in the Mayanur district children is given, and compared with that found in 3 South Indian towns (Aykroyd and Rajagopal, *loc. cit.*). It will be

noted that the incidence of these conditions was *less* than that observed in the previous investigation.

TABLE III.

*Incidence of phrynoderma, angular stomatitis, and Bitot's spots.*

Area.	Number examined.	Number showing phrynoderma.	Percentage.	Number showing angular stomatitis.	Percentage.	Number showing Bitot's spots.	Percentage.
Mayanur district	323	1	0.3	23	6.6	14	3.8
Coonoor ..	777	67	8.6	68	8.7	16	2.1
Mettupalayam ..	377	29	7.7	48	12.7	21	5.6
Calicut ..	426	2	0.5	43	10.1	35	8.2

## DISCUSSION.

How far are the diets of the various families sufficient in quantity? It is of little use to compare observed calorie intake with 'generally accepted standards', since such standards are European or American in origin. Only 4 families out of 44 consumed the 3,000 calories per consumption unit which are traditionally the requirement of 'an average man at average work'.

The families in Group I had not enough to eat. We know this, because they told us so. A complaint of hunger is perhaps better evidence of insufficient calorie intake than a textbook deduction. Average calorie intake in this group per consumption unit per family was 1,664; range 2,026 to 1,184. We may, therefore, take it that the daily calorie requirements of groups of South Indian village families, assessed in terms of the conventional scale used here, exceed the mean intake of this group.

Two facts regarding these families may be mentioned in passing. We were informed that they displayed 'an obvious lack of energy and initiative' which scarcely seems remarkable in the circumstances. Some time after the inquiry the missionary who kindly co-operated wrote: 'I have been finding a condition of cedema in very young children and have come to the conclusion that it is due to excess of crude salt. The salt is not cooked in the rice but sprinkled over it when the rice is being eaten. These children have improved at once when salt-free diet has been given'. It is possible that these children had a tendency towards cedema related to the 'hunger' or 'famine' cedema so commonly observed in Central and Eastern Europe during and just after the war."

The mean calorie intake of Group III is obviously no guide to the requirements of South Indian population groups, since the series included families with 'hungry mouths'. Calorie requirements can be deduced from intake only when intake is unrestricted by poverty or other circumstances. It is worth noting, however, that mean calorie intake in this group was greater than the mean intake (1,940) of 16 peasant families studied by Nicholls (*loc. cit.*) in Ceylon and that of 15 Javanese families, which was 2,116 (Ochese *et al.*, *loc. cit.*). Nicholls suggests that 'a diet of the value of about 2,200 calories is sufficient for the requirements of an agricultural labourer belonging to the smaller races of the Tropics, provided the diet is well balanced in necessary constituents'.

Five families in our investigation stood out from the rest in being in the possession of an income obviously sufficient to allow appetite to dictate the amount of food intake. The calorie intake of these was as follows:—

GROUP II, family	1	: 2,473
GROUP III	13	: 2,453
GROUP IV	1	: 2,394
GROUP IV	2	: 2,963
GROUP IV	3	: 2,464

---

Mean : 2,549

The mean figure is very much below that given by Wilson *et al.* (*loc. cit.*) as average intake per 'mean value' in 10 well-to-do Bengali Hindu families (3,411). These workers used Lusk's scale.

We have attempted, in a rough and ready fashion, to work out the minimum energy expenditure budget of a South Indian peasant. According to Rahman (1936) the average basal heat production per hour of a group of male students in Hyderabad was about 60 calories, some 7 to 8 per cent below American standards. Mason and Benedict (1931) recorded an average of 44 calories in 54 Indian women (students and teachers) in Madras—an average deviation from the Aub and du Bois standards of 17.2 per cent.

During sleep the metabolism rate has been reckoned as 10 per cent below the basal. Experiments on European and American subjects have shown that any form of manual labour raises metabolism at least 3 times above the basal, while 'sitting at rest' raises it about 1.3 times. Assuming that the South Indian peasant spends 8 hours of the 24 in sleep, 8 hours at work, and 8 hours 'sitting at rest', his energy expenditure budget would work out as follows:—

8 hours sleep at ..	..	54 calories per hour :	432 calories.
8 .. work at ..	..	180 ..	1,440 ..
8 .. 'sitting at rest'	..	86 ..	688 ..
Total :			2,560 ..

This seems a minimum budget, since expenditure at work is reckoned at the figure corresponding to expenditure in light manual occupations (e.g., carpentry). No figures are available for the energy expenditure involved by agricultural labour,

but in general it has been found that agricultural labourers in Europe and America have a high calorie intake. For example, the average calorie intake per consumption unit of a series of Roumanian peasant families was found to exceed 3,000 (Aykroyd, Alexa and Nitzulescu, 1935). The item of 688 calories for 8 hours 'sitting at rest' seems also a minimum, since peasants do not occupy all the waking hours not spent at work in this fashion.

Reckoned on similar basis, the daily energy expenditure of a woman with a basal metabolism rate per hour of 45 calories would work out at 1,900 calories. No data exist about the basal metabolism of Indian children.

All things considered, we are inclined to estimate the *minimum* daily calorie requirements per consumption unit of South Indian peasant families as lying in the neighbourhood of 2,500. If the diet of a labourer falls much below this figure, adjustment occurs as follows: basal metabolism is reduced, the body functioning, as it were, at a lower level of vitality, and energy output is necessarily made consonant with energy intake, since energy cannot be created. In simple terms, the under-fed labourer is lethargic and his output of work is small. A level of food intake which permits only a languid existence cannot be described as normal.

We are making use of the figure 2,500 in practical nutrition work, e.g., in drawing up diet schedules for institutions. It represents, of course, an average, and all individual cases falling somewhat below it need not be regarded as under-fed. If this figure is accepted, it appears that calorie intake in Groups I and II (3 families in the latter group) is definitely insufficient. In Group III, the mean approaches the standard minimum requirement, but conceals (since the intake of a number of families was in excess of the minimum) under-nutrition in a considerable proportion of families. The statement seems justified that one-third to one-half of the group of 44 families studied did not consume enough food during the period of investigation.

Judged by conventional standards, protein and fat intake was low. There was an almost complete absence of protein and fat of animal origin. The level of protein and fat intake in the poorer families was largely dependent on the nature of the cereal consumed, the protein and fat content of millet being higher than that of rice. The very low level of fat intake in Group I is due to the fact that the diet was mainly composed of rice and insufficient in quantity. Little can be said regarding the adequacy of calcium intake, except that it falls below conventional requirements in Groups II, III, and IV. The inclusion of millet, particularly ragi, in a largely cereal diet greatly raises calcium content, but Ranganathan (1935) has observed that the calcium in ragi and cambu is not well assimilated by rats. Phosphorus intake appears adequate; it is, however, to be observed that a considerable loss of phosphorus occurs on cooking from diets largely based on rice. Intake of iron appears high in relation to the general qualitative insufficiency of the diet; this may be ascribed to the fact that the iron content of many foods analysed in the laboratories has been found to be high, possibly because they originated in a district where the soil is rich in iron. The problem of iron requirements has been complicated by the observation that the iron contained in many foods is partially 'unavailable'.

Vitamin A was lacking, or present in infinitesimal quantities, in the diet of 39 of the 44 families. The highest intake, 500 $\gamma$  ( $\gamma=0.001$  mg.) per c.u. per day, was

that of family I, Group IV, which consumed milk products in reasonable quantities. Carotene intake in Groups I, II, and III, and vitamin-A and carotene intake in the families comprising Group IV, were estimated as follows:—

GROUP.	Mean carotene intake ( $\gamma$ per c. u. per day).	Range ( $\gamma$ ).
I ..	669	102 to 1,052
II ..	632	283 to 1,363
III ..	828	182 to 3,544

GROUP.	Carotene intake ( $\gamma$ per c. u. per day).	Vitamin-A intake ( $\gamma$ per c. u. per day).
IV { Family 1	440	500
" 2	1,098	200
" 3	1,400	256

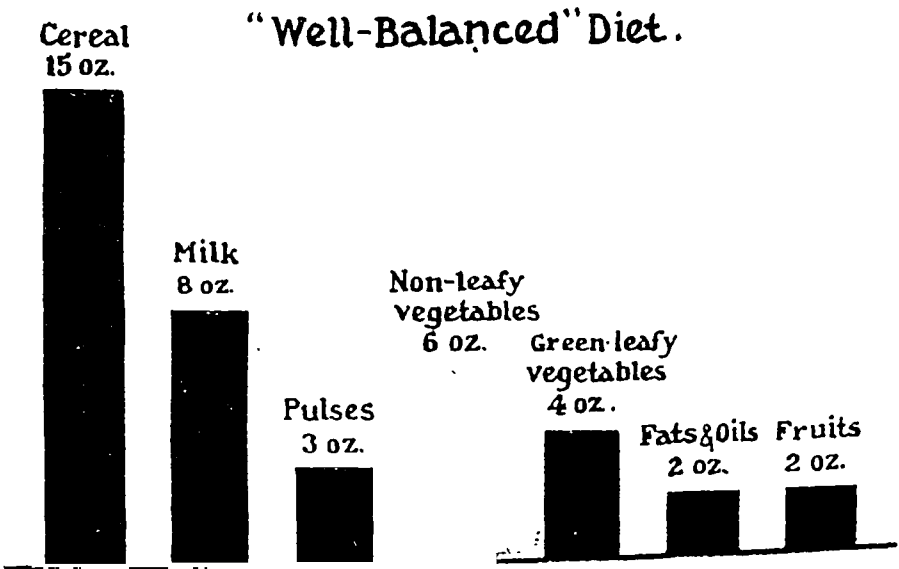
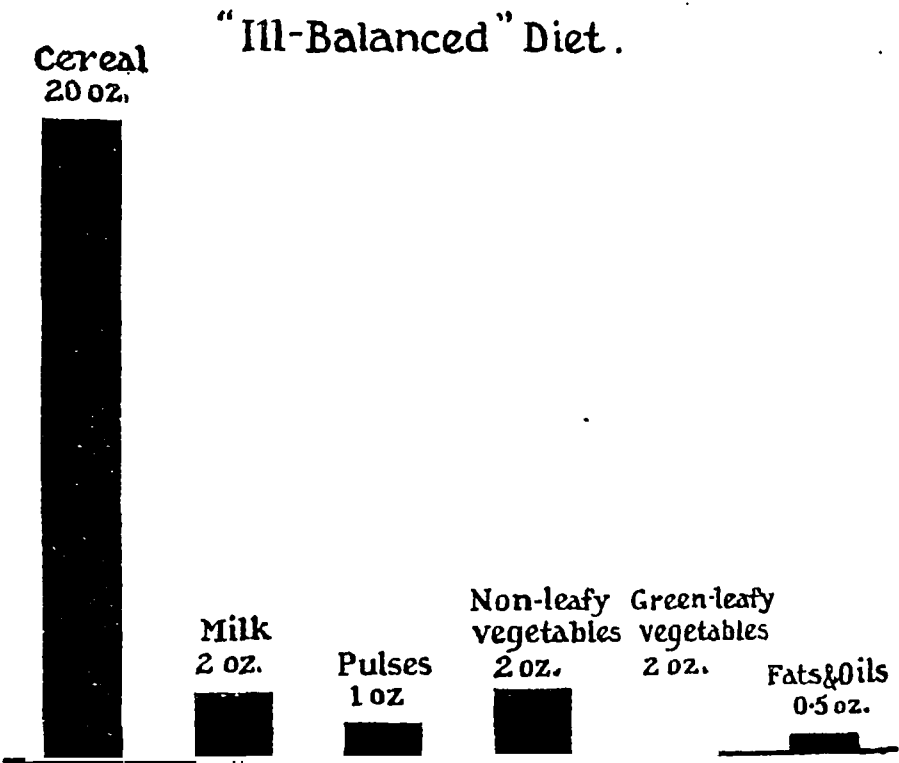
Estimates of vitamin-A and carotene intake are based on spectrographic assays carried out by Mr. N. K. De in these Laboratories. They are rough estimates, particularly as regards carotene, because the carotene content of foodstuffs is influenced by many factors (De, 1936), and the foodstuffs richest in carotene—green leafy vegetables—are those the intake of which it is most difficult to record accurately in diet surveys. In a previous paper (Aykroyd and Krishnan, 1936) we described the occurrence of xerophthalmia in a high percentage of children admitted to labour camps from a famine area, whose diet contained about 700 $\gamma$  of carotene daily. The view was expressed that this quantity is insufficient to cover the requirements of children. Bitot's spots were observed in 14 (3.8 per cent) of the group of children examined in the present investigation. We have found, in extensive investigations in children's hostels, that the percentage incidence of Bitot's spots and the apparent vitamin-A activity of the diet do not always run parallel, but in general this eye lesion appears to be associated with the consumption of a diet deficient in carotene and vitamin A. Both from the estimated quantities of these food factors consumed and the presence of xerophthalmia in the children examined, it may be concluded that the diet of the majority of families was somewhat deficient in vitamin-A activity.

Similarly, a deficiency of vitamin B<sub>2</sub> is shown by the presence of 'angular stomatitis'.

Beri-beri does not appear to be prevalent in the areas in which the surveys took place, though endemic in other parts of the Madras Presidency where raw milled rice is the chief staple. It has long been known that beri-beri is rarely or never seen in populations consuming home-pounded or milled parboiled rice, or in millet eaters. If very poorly fed village folk of the type investigated were to discard their parboiled rice or millet in favour of raw milled rice, beri-beri would doubtless

soon appear. Although no signs of scurvy were observed, many of the diets seem to be deficient in vitamin C.

CHART 2.





It is difficult to say how far the families studied were typical of South Indian peasants in general. To our collaborators in Chingleput and Mayanur they appeared typical of millions of village families throughout the country. It is clear that if Group I, which may without exaggeration be described as half-starved, is representative of a large group, the problem of under and malnutrition in South India is more serious than has yet been realized. Variations in diet at different seasons of the year require further study. We propose to carry out investigations at other times of the year on the groups studied here or on other similar groups.

There is reason to suppose that in India malnutrition is more marked among the poor of the towns and cities than among the poor of the countryside. The incidence of symptoms of food deficiency disease in the Mayanur district boys was lower than that in Coonoor, Mettupalayam and Calicut boys, and apparently also lower than that reported in Madras Corporation schools. It is our experience that 'state of nutrition' is better when the diet is based on millet, or at least contains a fair proportion of millet, than when the only cereal eaten is milled rice, as is often the case in towns and cities. Village populations have the further advantage over town populations that, when they do consume rice, they usually consume it in roughly milled form, often parboiled. The families in Group I, though they consumed home-pounded rice, were in a poor state of nutrition; their condition would be much worse if their staple cereal was highly milled. While living standards in India remain at the present level, we must view with alarm any extension of mechanical rice milling in rural areas.

Though in general the poor in the villages may suffer less severely from malnutrition than the urban poor, the present investigation emphasizes the deficiencies of the South Indian diet, notably the lack of milk and other 'protective' foods. Some months ago, a chart was prepared in the laboratory for propaganda purposes, illustrating, in terms of South Indian food habits, the difference between a 'well-balanced' and an 'ill-balanced' diet. The chart is reproduced (Chart 2) opposite.

Both diets as described would yield 2,600 calories, roughly adult daily requirements: the cost of the well-balanced diet, at current retail prices, is about Rs. 5 per adult per month; that of the ill-balanced diet, about Rs. 3-8. The diet of the families studied corresponds roughly in quality to the 'ill-balanced' diet, except that observed intake of milk and vegetables was even smaller than that shown diagrammatically. The chart may, however, be used to show the changes in agricultural production required to 'balance' the South Indian diet. Whether, given the existing standard of living and the fact of increasing population, changes in the direction of the well-balanced diet (e.g., a larger milk supply) are possible, is a fundamental problem which nutrition and agricultural research workers in India must face.

#### SUMMARY.

1. An investigation of the diet of various groups of villages in South India was carried out, the period of survey being 20 days. An account is given of the methods by which data were collected.

2. Intake of calories, proximate principles and minerals per consumption unit per day in the various families is tabulated and the composition of a number of characteristic diets as regards actual foodstuffs is described.

3. An account is given of the consumption of milk, meat, vegetables, fruit, and vegetable oils.

4. Examinations of a number of school children drawn from families whose diet was investigated, and similar families in the same district, were carried out. The height and weight averages of the children were compared with those of other malnourished groups in South India. The incidence of symptoms of deficiency disease was rather lower than that observed in various urban areas.

5. The adequacy of the calorie intake in the various groups is discussed and the tentative conclusion is reached that one-third to one-half of the families were under-fed. The various qualitative deficiencies in the diet are pointed out.

6. There is reason to suppose that less malnutrition is found in millet-eating areas than in rice-eating areas. The diet of rural populations is often better than that of town populations because the former consume unmilled rice or millet and the latter usually highly milled rice.

In conclusion, we must thank workers attached to the Indian Red Cross Society, Madras, the Church of Scotland Mission, Chingleput, and the Servants of India Society Rural Centre, Mayanur, for their painstaking and enthusiastic co-operation in these investigations.

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# SURVEY OF THE NUTRITIVE VALUE OF INDIAN FOODSTUFFS.

## Part I.

### THE CHEMICAL COMPOSITION OF 200 COMMON FOODS.

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DURING recent years increasing attention has been given to the problem of nutrition in India, and its importance is now generally realized. Knowledge of food values is the essential basis of almost all public health nutrition work; there is a considerable demand for such knowledge, not only on the part of medical and public health workers, but also on the part of the educated public. The rational planning of diets in institutions, for example, is impossible without the help of an authoritative table of food values. Further, such data are of importance in connection with the formulation of agricultural policies designed to improve the diet of the people. Finally, detailed knowledge of food values is required for developing the research programme of the laboratories, which includes diet surveys throughout the country.

Up to the present no comprehensive survey of Indian food values has been carried out. McCay (1910), Sahasrabuddhe (1925), and Stewart *et al.* (1931) have published tables based on the chemical analysis of some common Indian foods; their work does not, however, cover a wide range. McCarrison's (1929) primer, 'Food', which has been widely used in planning diet schedules, provides data about only a limited number of foods, and includes no information about mineral content. In certain other Eastern countries complete and authoritative tables are already in existence; mention may be made of Rosedale's (1935) 'Chemical Analysis of Malayan Foods' and Hermans's (1932) 'Food Values', published by the Philippine Bureau of Science. Considerable attention is being given to the same subject in China and Ceylon.

The present investigation forms part of a systematic foodstuffs survey now proceeding at the Coonoor Laboratories. It deals with the results of the chemical analysis of some 200 foodstuffs, covering most of the cereals and pulses, vegetables (leafy, roots, and tubers, and others), nuts and oil seeds, fruits, condiments and spices, and a few flesh foods in common use.

#### COLLECTION OF MATERIAL.

The foodstuffs analysed were mostly obtained in the local market. Foods which may be described as common Indian foods, consumed throughout the country, originated in the majority of cases in the neighbouring plains of the Coimbatore district; others of a kind less widely used in India (e.g., European vegetables, such as lettuce) were largely grown in the neighbourhood of Coonoor, 6,000 feet above sea-level. Among the foods analysed were some from other parts of India, including North India. The place of origin of each foodstuff is indicated in column 3 of the tables. The edible portion of the foodstuff, in as fresh a state as possible, was used for the analysis; the results of analysis relate only to the edible portion.

#### METHODS OF ANALYSIS.

The chemical analysis of the foodstuffs includes a determination of the following: moisture, the three 'proximate principles' (protein, fat, and carbohydrate), total mineral matter, crude fibre, and the three minerals usually considered of importance in practical dietetics: calcium, phosphorus, and iron. The methods employed were mostly standard ones and, briefly described, were as follows:—

1. *Sampling*.—About 100 grammes to 150 grammes of the foodstuff, freed as far as possible from extraneous matter, are ground to a fine powder and passed through a fine-mesh sieve. Where such powdering cannot be done, as in the case of fruits, vegetables, etc., a representative sample is obtained, and fractions used for the determination of the following constituents.

2. *Moisture*.—About 2 grammes to 3 grammes of the test sample are dried to constant weight in an electric hot-air oven maintained at 100°C., and the loss in weight consequent on drying is reckoned as moisture.

3. *Total mineral matter*.—About 25 grammes to 100 grammes of the test material, the amount depending on the nature of the foodstuff, are heated slowly in a platinum dish over a Bunsen flame till complete carbonization takes place, then removed to an electric muffle furnace and ashed to constant weight. The ashing was carried to the stage at which there were no enclosed carbon particles. The weight of ash is reckoned as total mineral matter.

4. *Ether extractives*.—Five grammes to 20 grammes of the test material are weighed into a fat-free thimble slowly dried in a hot-air oven till fairly dry. It is then extracted with dry ether for about 20 to 24 hours in a Soxhlet's apparatus with ground-glass joints. The extract is freed from the solvent, dried to constant weight, and reckoned as ether extractives.

5. *Crude fibre*.—The residue left over from (4) is utilized for the determination of crude fibre, the method being that adopted by the Association of Official Agricultural Chemists (1930).

6. *Proteins*.—Total nitrogen is determined by the usual Kjeldahl's method and the protein content calculated therefrom by multiplying the nitrogen figure by 6.25.

7. *Carbohydrates*.—The carbohydrate content is obtained by subtracting the sum of moisture, total mineral matter, ether extractives, crude fibre, and proteins, from 100.

8. *Calorific value*.—This is obtained by multiplying the sum of carbohydrates and proteins by 4 and adding to it the figure obtained by multiplying the ether extractives by 9,

i.e., calorific value = 4 (carbohydrates + proteins) + 9 × ether extractives.

9. *Fractional analysis of the ash*.—The ash obtained under (3) is gently moistened with a few drops of distilled water and then 5 c.c. to 10 c.c. of pure, iron-free hydrochloric acid are added. The solution is boiled for a few minutes and filtered into a 100 c.c. measuring flask. The filtrate and the washings of the filter-paper, collected in the same flask, are made up to mark; aliquots are taken for the determination of calcium, phosphorus, and iron.

10. *Calcium*.—Ten c.c. to 25 c.c. of the solution prepared as under (9) are rendered strongly alkaline with ammonia. Ammonium oxalate is then added in excess and the solution brought to the boil. While still hot, the solution is rendered strongly acid with glacial acetic acid. The precipitate of calcium oxalate is allowed to settle over-night. It is then washed till free from ammonium oxalate with boiling distilled water, dissolved in hot, dilute sulphuric acid, and titrated against deci- or centi-normal potassium permanganate. Whatman No. 44 filter-paper is used.

11. *Phosphorus*.—The volumetric method (Official) recommended by the Association of Official Agricultural Chemists (*loc. cit.*) is employed. Ten c.c. to 25 c.c. of the solution described under (9) are used for this estimation. Three per cent potassium nitrate solution is used for washing the precipitate of phosphomolybdate, instead of distilled water.

12. *Iron*.—Iron is estimated colorimetrically by the potassium thiocyanate method, the colour obtained being taken up by extra pure amyl alcohol (Farrar, Jr., 1935). The solution prepared as under (9) is employed except in the case of vegetables, fruits, etc., with acid juices, which have, in the preparation of the original sample, been cut with a steel knife. In such cases about 5 grammes of the material, obtained without making use of any metallic blades, are ashed, and the ash brought to solution with iron-free hydrochloric acid; an aliquot of this solution is taken for iron estimation. To this aliquot, taken in a separating funnel, a drop or two of concentrated, iron-free nitric acid is added to oxidize any ferrous salt. Five c.c. of amyl alcohol are then added, followed by 5 c.c. of 20 per cent potassium thiocyanate solution. The liquid is shaken well and the amyl alcohol layer separated off; this extraction of the thiocyanate colour is done more than once, usually thrice; the amyl alcohol layers in the different extractions are mixed, made up to volume in either a 10 c.c. or a 25 c.c. measuring flask, depending on the intensity of the colour, and matched in a colorimeter against suitable standards treated likewise. Blank estimations are carried out during each set of iron determinations to ensure the freedom of the reagents employed from iron, as even reagents labelled 'extra pure' not seldom contain traces of iron.

TABLE Ia.  
Cereals.

Name.	Botanical name.	Source.	Moisture per cent.	Protein per cent.	Ether extractives per cent.	Mineral matter per cent.	Fibre per cent.	Carbohydrates per cent.	Calcium (Ca) per cent.	Phosphorus (P) per cent.	Iron (Fe) mg. per cent.	Calorific value per 100 g.	REMARKS.
1	2	3	4	5	6	7	8	9	10	11	12	13	14
Arrow-root flour (West Indian).	<i>Maranta arundinacea</i>	Local bazaar	16.51	0.17	0.06	0.12	..	83.14	0.009	0.017	1.00	333.8	
'Bajra' or 'cambu'	<i>Pennisetum typhloideum</i>	Coimbatore	12.41	11.59	4.90	2.95	1.22	67.14	0.049	0.352	8.84	359.9	
Barley ..	<i>Hordeum vulgare</i>	Local	12.48	11.47	1.28	1.51	3.92	69.34	0.025	0.225	3.70	334.8	
'Cholam'	<i>Sorghum cernuum</i>	Bellary	12.31	10.10	1.89	1.18	..	74.52	0.014	0.246	7.44	355.5	
" yellow	<i>S. durra</i>	Coimbatore	11.65	8.84	1.47	1.56	..	76.48	0.040	0.309	6.10	344.5	
" 'irungu'	"	"	12.12	10.51	2.96	1.56	..	..	0.017	0.300	5.72	..	
" 'irungu'	<i>S. vulgare</i>	"	11.42	12.23	1.50	2.73	..	72.12	0.038	0.274	5.47	350.9	
Italian millet	<i>Setaria Italica</i>	Local bazaar	12.31	12.31	4.65	3.24	7.95	60.62	0.026	0.287	6.25	333.6	
'Koota'	<i>Fagopyrum esculentum</i>	Bijoor (U. P.)	11.33	10.26	2.48	2.42	8.60	65.03	0.068	0.299	13.20	322.4	
Maize, Indian corn (tender green).	<i>Zea mays</i>	Local	79.36	4.33	0.46	0.65	..	15.20	0.005	0.102	0.68	82.3	Without outer husk.
'Mukhana'	—	Bijoor (U. P.)	12.75	9.70	0.14	0.52	..	76.89	0.016	0.091	1.38	347.6	
Oatmeal	<i>Avena sativa</i>	Local bazaar	10.68	13.55	7.59	1.78	3.46	62.94	0.045	0.384	3.81	374.3	
'Pani varagu'	<i>Panicum miliaceum</i>	Coimbatore	11.85	12.49	1.06	3.39	2.16	69.05	0.014	0.333	5.72	335.7	
'Ragi' ..	<i>Eleusine coracana</i>	Mangalore	13.34	6.94	1.11	2.19	..	76.42	0.358	0.238	5.40	343.4	
" brown	"	Coimbatore	12.58	7.92	1.54	2.21	..	75.75	0.386	0.311	6.22	348.5	
" 'marua'	"	Darbhanga	13.22	6.44	1.22	2.33	..	76.79	0.257	0.268	4.53	343.9	
Rice, 'guddu'	<i>Oryza sativa</i>	Mangalore	12.19	9.06	0.41	0.67	..	77.75	0.007	0.104	5.57	351.0	
" 'maskoti'	"	"	12.20	7.83	0.25	0.85	..	79.07	0.004	0.165	4.65	349.1	
" 'suggi'	"	"	12.19	8.49	0.35	0.70	..	78.27	0.005	0.134	2.75	350.2	
" ..	"	"	12.63	8.50	0.39	0.86	..	77.62	0.010	0.277	6.14	349.0	
" raw, polished ..	"	Local bazaar	12.96	6.85	0.55	0.50	..	79.14	0.007	0.108	1.02	348.9	
" parboiled	"	"	13.29	6.44	0.64	0.77	..	78.86	0.007	0.149	2.22	347.0	
" white 'puttu'	"	"	12.97	7.51	0.37	0.40	..	78.75	0.008	0.081	3.33	348.4	
" black 'puttu'	"	"	12.26	7.69	1.27	1.25	0.65	76.88	0.012	0.241	4.93	349.7	
" beaten	"	"	12.23	6.63	1.15	1.73	..	78.26	0.016	0.217	8.00	349.9	
" puffed	"	"	14.68	7.46	0.12	3.41	..	74.33	0.024	0.162	6.15	329.1	
Sago ..	<i>Metroylon sago</i>	Local	12.20	0.24	0.17	0.30	..	87.09	0.010	0.006	1.27	350.9	
'Sannu'	<i>Panicum miliare</i>	"	11.46	7.70	4.71	4.83	7.60	63.70	0.015	0.364	7.08	328.0	
'Sannu', millet	<i>Panicum crusgalli</i> var. <i>frumentaceum</i> .	Coimbatore	11.87	6.22	2.23	4.44	9.83	65.41	0.019	0.282	2.86	306.6	
'Singham', dry ..	<i>Trapa bispinosa</i>	Bijoor (U. P.)	13.76	13.43	0.83	3.07	..	68.91	0.069	0.143	2.35	336.8	
Vernicelli	<i>Paspalum scrobiculatum</i>	Coimbatore	11.97	8.66	0.44	3.07	..	78.76	0.020	0.079	0.33	353.6	
'Varagu' or 'kodu' ..	"	"	12.77	8.31	1.37	2.92	8.97	65.66	0.039	0.242	5.17	308.2	
Wheat ..	<i>Triticum vulgare</i>	Mangalore	12.77	11.77	1.45	1.49	1.20	71.30	0.054	0.315	5.34	345.4	

TABLE 1b.

## Pulses.

Name.	Botanical name.	Source.	Moisture per cent.	Protein per cent.	Ether extractives per cent.	Mineral matter per cent.	Fibre per cent.	Carbohydrates per cent.	Calcium (Ca) per cent.	Phosphorus (P) per cent.	Iron (Fe) mg. per cent.	Calorific value per 100 g.	REMARKS.
I	2	3	4	5	6	7	8	9	10	11	12	13	14
Bengal gram ..	<i>Cicer arietinum</i>	Local bazaar	9.83	17.08	5.26	2.68	3.92	61.23	0.185	0.236	9.83	360.6	With husk.
" ..	"	Mangalore	12.17	16.19	4.36	3.01	3.92	60.35	0.194	0.444	9.83	345.4	"
Bengal gram, roasted	"	Local bazaar	11.17	22.54	5.17	2.19	..	58.93	0.067	0.311	8.89	372.4	Without outer husk.
' Bhettans ' ..	<i>Glycine hispida</i>	Darbanga	8.80	41.29	16.95	4.49	4.25	24.22	0.213	0.603	9.88	414.6	
Black gram ..	<i>Phaseolus mungo</i>	Local bazaar	10.87	23.95	1.38	3.39	..	60.41	0.200	0.367	9.80	358.7	Without outer husk.
Cow gram ..	<i>Vigna catiung</i>	"	12.00	24.56	0.69	3.23	3.75	55.77	0.073	0.485	3.81	327.5	
" ..	"	Coimbatore	11.72	23.63	1.06	3.02	3.75	50.82	0.081	0.387	6.67	331.3	
Field bean, dry ..	<i>Dolichos lablab</i>	Mysore	9.60	24.94	0.78	3.21	1.38	60.09	0.063	0.446	1.98	347.1	
" ..	"	Local bazaar	12.21	20.43	0.84	2.90	1.38	62.24	0.113	0.334	5.33	338.2	
Green gram ..	<i>Phaseolus radiatus</i>	"	10.42	23.96	1.26	3.57	4.10	56.69	0.143	0.281	8.40	342.8	With husk.
Horse gram ..	<i>Dolichos biflorus</i>	Mangalore	11.81	22.01	0.54	3.13	5.30	57.21	0.275	0.385	7.59	321.7	
' Khassai ' ..	<i>Cicer arietinum (Lathyrus sativa P.)</i>	Darbanga	9.98	28.22	0.57	2.99	..	58.24	0.107	0.496	5.63	351.0	
' Masur dual ' (lentil)	<i>Lens culcanta</i>	Local bazaar	12.44	25.10	0.74	2.13	..	59.56	0.130	0.250	1.98	345.4	
Peas, dried ..	<i>Pisum sativum</i>	"	16.03	19.68	1.14	2.12	4.50	50.53	0.068	0.298	4.44	315.1	
" roasted ..	"	"	9.90	22.94	1.39	2.34	..	63.43	0.033	0.357	5.00	358.0	
' Rajmah ' ..	—	Lahore	12.03	22.89	1.31	3.22	..	60.55	0.256	0.408	5.84	345.6	
' Ravan ' ..	<i>Vigna catiung</i>	"	12.67	23.40	1.28	2.92	..	59.73	0.075	0.430	4.31	344.0	
Red gram ..	<i>Cajanus indicus</i>	Local bazaar	15.23	22.27	1.71	3.56	..	57.23	0.136	0.264	8.80	341.9	Without outer husk.
" ..	"	Mangalore	12.76	21.12	1.84	2.80	..	61.48	0.056	0.276	8.80	347.0	
Soya bean ..	<i>Glycine hispida</i>	Ahmedabad	8.08	43.22	19.50	4.63	3.72	20.85	0.237	0.689	11.59	431.8	

TABLE Ic.

Vegetables : leafy.

Name.	1	2	3	4	5	6	7	8	9	10	11	12	13
		Botanical name.	Source.	Moisture per cent.	Protein per cent.	Ether extractives per cent.	Mineral matter per cent.	Fibre per cent.	Carbohydrates per cent.	Calcium (Ca) per cent.	Phosphorus (P) per cent.	Iron (Fe) mg. per cent.	Calorific value per 100 g.
Amaranth (tender) (spined)	..	<i>Amaranthus gangeticus</i>	Local Coimbatore	85.78	4.90	0.50	3.05	..	5.77	0.500	0.100	21.40	47.2
" (tender)	..	<i>A. spinosus</i>	"	85.00	3.00	0.25	3.61	..	8.14	0.800	0.052	22.91	46.8
Bamboo (tender shoots).	..	<i>Bambusa arundinacea</i>	Local	87.06	3.92	0.09	1.40	..	7.53	0.016	0.088	0.11	46.6
Brussels sprouts	..	<i>Brassica oleracea bullata gemmifera.</i>	Local	84.57	4.68	0.46	1.02	..	9.27	0.045	0.082	2.33	59.9
Cabbage	..	<i>Brassica oleracea capitata</i>	"	90.20	1.75	0.11	0.61	0.95	6.38	0.034	0.046	0.76	33.5
Celery	..	<i>Apium graveolens rapaceum.</i>	"	81.27	5.97	0.61	2.11	1.42	8.62	0.226	0.137	6.25	63.9
Coriander	..	<i>Coriandrum sativum</i>	"	87.90	3.20	0.64	1.65	..	6.52	0.137	0.058	9.97	45.0
Curry leaves	..	<i>Murraya koenigii</i>	Coimbatore	66.31	6.11	1.04	4.17	6.37	16.00	0.811	0.057	3.09	97.8
Drumstick	..	<i>Moringa oleifera</i>	"	74.98	6.65	1.73	2.27	0.87	13.50	0.437	0.071	7.00	96.2
Feenugreek	..	<i>Trigonella foenum-graecum</i>	Local	81.78	4.86	0.86	1.57	1.04	9.89	0.472	0.047	16.90	66.7
Garden cress	..	<i>Lepidium sativum</i>	"	82.30	5.79	0.96	2.16	..	8.79	0.359	0.113	28.57	67.0
'Gogu'	..	<i>Hibiscus sabdariffa</i>	Godavari	86.22	1.70	1.07	1.02	..	9.99	0.181	0.038	5.38	56.4
Ipomoea	..	<i>Ipomoea reptans</i>	Local	90.33	2.90	0.40	2.11	..	4.26	0.113	0.048	3.90	32.2
Lettuce	..	<i>Lactuca sativa</i>	"	92.94	2.05	0.26	1.18	0.53	3.04	0.051	0.030	2.39	22.7
Mint	..	<i>Mentha viridis</i>	"	82.99	4.81	0.59	1.56	1.95	8.10	0.204	0.077	15.56	57.0
'Neem' (mature)	..	<i>Azadirachta indica</i>	Coimbatore	59.44	7.09	1.04	3.36	6.19	22.88	0.514	0.080	17.14	129.2
" (tender)	..	"	"	59.36	11.56	2.97	2.61	2.22	21.28	0.127	0.189	25.30	158.1
Parsley	..	<i>Petroselinum sativum</i>	Local	68.42	5.93	0.97	3.17	1.80	19.71	0.392	0.195	17.86	111.3
'Agathi'	..	<i>Sesbania grandiflora</i>	Coimbatore	76.73	8.40	1.39	3.11	2.17	11.80	1.131	0.077	3.91	93.3
'Mamthakali'	..	<i>Solanum nigrum</i>	Local	82.12	5.86	1.00	2.13	..	8.89	0.410	0.074	20.51	68.0
Spinach	..	<i>Spinacia oleracea</i>	Godavari	91.65	1.92	0.85	1.50	..	4.07	0.061	0.010	4.95	31.6



TABLE Id.

## Vegetables: roots and tubers.

Name.	Botanical name.	Source.	Moisture per cent.	Protein per cent.	Ether extractives per cent.	Mineral matter per cent.	Fibre per cent.	Carbohydrates per cent.	Calcium (Ca) per cent.	Phosphorus (P) per cent.	Iron (Fe) mg. per cent.	Calorific value per 100 g.	REMARKS.
1	2	3	4	5	6	7	8	9	10	11	12	13	14
Beet root ..	<i>Beta vulgaris</i>	Local	83.81	1.65	0.05	0.75	..	13.74	0.195	0.057	0.98	62.0	Botanical name could not be traced.
Carrot ..	<i>Daucus carota</i>	"	86.00	0.92	0.07	1.08	1.18	10.75	0.082	0.030	1.52	47.3	
Colocasia ..	<i>Colocasia antiquorum</i>	"	73.05	2.95	0.10	1.72	..	22.18	0.038	0.143	2.13	101.4	
' Onthalai gasu ' * ..	—	"	84.40	1.24	0.05	0.34	..	13.97	0.013	0.024	0.53	61.3	
Paranip ..	<i>Pastinaca sativa</i>	"	72.41	1.33	0.27	1.12	1.68	23.19	0.048	0.041	0.40	100.5	
Potato ..	<i>Solanum tuberosum</i>	"	74.73	1.73	0.13	0.61	..	22.80	0.004	0.034	0.68	99.3	Botanical name could not be traced.
Radish (pink) ..	<i>Raphanus sativus</i>	"	90.76	0.61	0.32	0.85	..	7.46	0.046	0.017	0.47	35.2	
" (white) ..	" "	"	94.41	0.70	0.06	0.68	..	4.25	0.054	0.025	0.40	20.3	
Sweet potato ..	<i>Ipomoea batatas</i>	Local bazaar	66.51	1.24	0.32	1.04	..	30.89	0.017	0.050	0.79	131.4	
' Tapioca	<i>Manihot utilisima</i>	"	59.37	0.68	0.20	0.98	..	38.77	0.045	0.039	0.92	159.6	
Yam, elephant ..	<i>Amorphophallus cam-panulatus.</i>	"	78.79	1.24	0.02	0.82	0.80	18.33	0.046	0.022	0.62	78.4	Botanical name could not be traced.
" ordinary ..	<i>Typhonium trilobatum</i>	"	69.90	1.41	0.09	1.55	..	28.46	0.061	0.016	1.30	120.3	

\* A root resembling tapioca.

TABLE Ie.  
Other vegetables.

Name.	Botanical name.	Source.	Moisture per cent.	Protein per cent.	Ribber extractives per cent.	Mineral matter per cent.	Ribre per cent.	Carbohydrates per cent.	Calcium (Ca) per cent.	Phosphorus (P) per cent.	Iron (Fe) mg. per cent.	Caloric value per 100 g.
1	2	3	4	5	6	7	8	9	10	11	12	13
Amaranth stem ..	<i>Anaranthus gangeticus</i>	Local	92.49	0.85	0.11	1.84	1.20	3.51	0.263	0.034	1.80	18.4
Artichoke ..	<i>Cynara scolymus</i>	"	77.27	3.64	0.15	1.75	1.20	15.99	0.120	0.101	2.29	79.9
Ash gourd ..	<i>Benincasa cerifera</i>	Coimbatore	96.01	0.41	0.06	0.34	..	3.18	0.025	0.017	0.45	14.9
Bitter gourd ..	<i>Momordica charantia</i>	"	92.39	1.64	0.15	0.76	0.80	4.26	0.023	0.065	2.22	25.0
" (small variety)	"	W. Godavari	83.16	2.87	0.98	1.43	1.70	9.86	0.045	0.144	9.35	59.7
Brinjal ..	<i>Solanum melongena</i>	Coimbatore	91.49	1.31	0.26	0.50	..	6.44	0.024	0.064	1.31	33.3
Broad beans ..	<i>Dolichos lablab</i> var. <i>lignosus</i> .	"	82.41	4.49	0.12	0.98	2.02	9.98	0.045	0.055	1.61	59.0
Calabash cucumber	<i>Lagenaria vulgaris</i>	"	96.34	0.16	0.14	0.49	..	2.87	0.015	0.003	0.69	13.4
Cauliflower ..	<i>Brassica oleracea botrytes</i>	Local	89.43	3.51	0.41	1.36	..	5.29	0.034	0.061	1.25	38.9
'Cho-cho' marrow	<i>Sechium edule</i>	"	92.53	0.70	0.11	0.41	..	6.25	0.138	0.026	0.62	28.8
Celery stalks ..	<i>Apium graveolens rapaceum</i> .	"	93.53	0.79	0.09	0.89	1.17	3.53	0.033	0.038	4.76	18.1
Cluster beans ..	<i>Cyamopsis psoralitoides</i>	Coimbatore	82.45	3.67	0.17	1.35	2.26	10.10	0.129	0.051	5.76	56.6
Colocasia stems ..	<i>Colocasia antiquorum</i>	"	93.44	0.34	0.34	1.20	0.59	4.09	0.062	0.024	0.50	20.8
Cucumber ..	<i>Cucumis sativus</i>	"	96.42	0.45	0.06	0.31	..	2.76	0.012	0.025	1.48	13.4
" ..	"	Godavari	90.86	0.72	0.13	0.93	..	7.36	0.037	0.062	2.12	33.5

	Local	73-75	8-28	0-27	0-94	..	16-76	0-036	0-142	2-27	102-6
Double beans	..										
<i>Faba vulgaris</i>	Local										
Drumstick	..	86-88	2-53	0-11	1-96	4-80	3-72	0-032	0-111	5-28	26-0
<i>Moringa oleifera</i>	Coimbatore										
French beans	..	91-43	1-74	0-11	0-63	1-80	4-30	0-048	0-025	1-67	25-5
<i>Phaseolus vulgaris</i>	Local										
Indian gooseberry	..	81-21	0-52	0-09	0-68	3-42	14-08	0-045	0-019	1-23	59-2
<i>Emblic officinalis</i>	Coimbatore										
Ipomoea stems	..	93-67	0-83	0-20	1-76	..	3-54	0-081	0-027	0-78	19-3
<i>Ipomoea repans</i>	Local										
Jack (tender)	..	84-01	2-61	0-31	0-01	2-78	9-38	0-026	0-043	1-66	50-8
<i>Artocarpus integrifolia</i>	Coimbatore										
Jack-fruit seeds	..	51-60	6-60	0-36	1-54	1-45	38-45	0-049	0-133	0-16	183-4
"	"										
<i>Solanum xanthocarpum</i>	"	75-50	3-09	0-77	1-57	14-15	4-02	0-100	0-091	1-22	39-0
* Kandan kathiri' ..											
' Kovai ' fruit (raw)	Bangalore	93-07	1-17	0-10	0-46	1-56	3-64	0-037	0-030	1-35	20-1
<i>Coccinia indica</i>											
Knoi-khol	..	92-10	1-07	0-20	0-65	..	5-08	0-023	0-035	0-40	30-0
<i>Brassica oleracea caulorapa</i>	Local										
Lady's fingers	..	87-95	2-20	0-20	0-74	1-15	7-76	0-094	0-078	1-54	41-6
<i>Hibiscus esculentus</i>	Coimbatore										
Leeks ..	..	78-91	1-84	0-10	0-68	1-32	17-15	0-052	0-073	2-30	76-9
<i>Allium porum</i>	Local										
Mango (green)	..	90-03	0-65	0-11	0-37	..	8-84	0-012	0-017	4-48	39-0
<i>Mangifera indica</i>	"										
Nat of 'avocado' pear.	"	63-67	2-32	0-71	1-10	..	32-00	0-018	0-083	1-19	144-5
<i>Persea drymifolia</i>											
Onion stalk	..	87-64	0-94	0-24	0-77	1-60	8-81	0-048	0-054	7-50	41-2
<i>Allium cepa</i>	"										
Peas (English)	..	72-09	7-18	0-12	0-75	..	19-86	0-019	0-078	1-47	109-2
<i>Pisum sativum</i>	"										
Pink beans	..	88-49	2-44	0-18	0-58	2-10	6-21	0-035	0-039	1-24	36-2
<i>Phaseolus vulgaris</i>	"										
Plantain flower	..	90-22	1-45	0-19	1-21	1-93	5-00	0-034	0-048	0-12	27-5
<i>Musa paradisica</i>	"										
Plantain (green)	..	83-24	1-41	0-23	0-51	..	14-61	0-005	0-027	0-60	66-2
"	Coimbatore										
Plantain stem	..	88-26	0-48	0-05	0-61	0-76	9-84	0-009	0-005	1-06	41-7
"	"										
Pumpkin	..	92-60	1-36	0-07	0-58	..	5-39	0-009	0-030	0-67	27-6
<i>Cucurbita maxima</i>	"										
Rhubarb stalks	..	92-74	1-10	0-51	1-05	0-85	3-75	0-119	0-012	2-20	24-0
<i>Rheum Rhabonticum</i>	Local										
Ridge gourd	..	95-43	0-52	0-12	0-34	..	3-59	0-035	0-040	1-58	17-5

TABLE 1e—concl.

## Other vegetables—concl.

Name.	Botanical name.	Source.	Moisture per cent.							Fibre per cent.	Carbohydrates per cent.	Calcium (Ca) per cent.	Phosphorus (P) per cent.	Iron (Fe) mg. per cent.	Calorific value per 100 g.
			4	5	6	7	8	9	10	11	12	13			
1	2	3													
Snake gourd ..	<i>Trichosanthes anguina</i>	Coimbatore	94.06	0.46	0.31	0.70	..	4.47	0.045	0.024	1.28	22.5			
Spinach stalks ..	<i>Spinacia oleracea</i>	Local	93.37	0.87	0.14	1.82	..	3.80	0.090	0.015	1.34	19.9			
'Sundakai' (dry) ..	<i>Solanum torvum</i>	Local bazaar	12.25	8.31	1.66	5.09	17.58	55.11	0.370	0.179	22.22	268.6			
Tomato (green) ..	<i>Lycopersicum esculentum</i>	Local	92.81	1.88	0.06	0.68	..	4.57	0.016	0.039	2.37	26.3			
Turnip ..	<i>Brassica rapa</i>	"	91.10	0.52	0.23	0.60	..	7.52	0.028	0.035	0.42	34.2			
Vegetable marrow ..	<i>Cucurbita pepo</i>	Coimbatore	94.84	0.48	0.09	0.27	..	4.32	0.002	0.026	0.63	20.0			

TABLE If.

## Oil-seeds and nuts.

Name.	Botanical name.	Source.	Moisture per cent.	Protein per cent.	Ether extractives per cent.	Mineral matter per cent.	Fibre per cent.	Carbohydrates per cent.	Calcium (Ca) per cent.	Phosphorus (P) per cent.	Iron (Fe) mg. per cent.	Calorific value per 100 g.
1	2	3	4	5	6	7	8	9	10	11	12	13
Almond ..	<i>Prunus amygdalis</i>	Local bazaar	5.23	20.75	58.92	2.90	1.70	10.50	0.225	0.492	3.47	655.3
Cashew ..	<i>Anacardium occidentale</i>	"	5.89	21.19	46.63	2.43	1.27	22.29	0.053	0.449	4.95	596.3
Coco-nut ..	<i>Cocos nucifera</i>	"	36.28	4.47	41.60	0.96	3.59	13.19	0.013	0.243	1.70	444.7
Gingelly ..	<i>Sesamum indicum</i>	"	5.08	18.33	43.26	5.20	2.88	25.25	1.453	0.574	10.54	563.7
Ground-nut ..	<i>Arachis hypogaea</i>	"	7.92	26.72	40.13	1.87	3.07	20.29	0.048	0.392	1.56	549.2
Ground-nut, roasted	"	"	4.04	31.54	30.76	2.28	3.07	19.31	0.045	0.435	0.29	561.2
Linseed ..	<i>Linum usitatissimum</i>	"	6.57	20.27	37.11	2.44	4.80	28.81	0.173	0.371	2.65	530.3
Mustard ..	<i>Brassica juncea</i>	"	8.46	22.04	39.64	4.19	1.80	23.87	0.488	0.704	17.88	540.4
Pistachios ..	<i>Pistacia vera</i>	"	5.58	19.81	53.51	2.75	2.10	16.25	0.136	0.431	13.70	625.8
Walnut ..	<i>Juglans regia</i>	"	4.53	15.64	64.49	1.84	2.60	10.90	0.095	0.384	4.76	686.6

TABLE Ig.

## Fruits.

Name.	Botanical name.	Source.	Moisture per cent.	Protein per cent.	Ether extractives per cent.	Mineral matter per cent.	Fibre per cent.	(Carbohydrates per cent.	Calcium (Ca) per cent.	Phosphorus (P) per cent.	Iron (Fe) mg. per cent.	Calorific value per 100 g.	REMARKS.
1	2	3	4	5	6	7	8	9	10	11	12	13	14
Apple ..	<i>Pyrus malus</i>	Local	85.94	0.31	0.08	0.27	..	13.40	0.013	0.021	1.68	55.0	Preserved.
Banana ..	<i>Musa sapientum</i>	Local bazaar	61.39	1.33	0.15	0.72	..	36.41	0.002	0.045	0.42	147.6	
Bullock's heart ..	<i>Anona reticulata</i>	Local	76.79	1.39	0.16	0.74	..	20.92	0.014	0.009	0.63	90.7	
Cape gooseberry ..	<i>Physalis peruviana</i>	"	82.69	1.78	0.17	0.59	3.19	11.58	0.006	0.058	1.80	55.0	
Dates (Persian) ..	<i>Phoenix dactylifera</i>	Local bazaar	26.11	3.04	0.16	1.31	2.07	67.31	0.067	0.076	10.58	282.8	
Figs ..	<i>Ficus carica</i>	"	80.80	1.31	0.23	0.64	..	17.02	0.055	0.026	1.16	75.4	
Grapes (blue variety)	<i>Vitis vinifera</i>	"	85.51	0.83	0.10	0.43	3.00	10.13	0.025	0.019	0.40	44.7	
Grape fruit (Triumph)	<i>Citrus grandis</i> var. <i>maxima</i> .	Local	92.04	0.71	0.03	0.20	..	7.02	0.017	0.021	0.18	31.2	
" (Marsh's seedless).	"	"	88.51	0.95	0.06	0.35	..	10.13	0.025	0.027	0.20	44.9	
Guava (country) ..	<i>Psidium guyana</i>	Coimbatore	76.07	1.46	0.19	0.81	6.90	14.57	0.013	0.040	1.04	65.8	
" (hill) ..	<i>P. catibianum</i>	Local	85.31	0.95	0.16	0.61	4.82	8.15	0.046	0.022	1.20	37.8	
Jack-fruit ..	<i>Artocarpus integrifolia</i>	Coimbatore	77.20	1.86	0.10	0.78	1.09	17.88	0.022	0.028	0.46	79.9	
Jambu fruit ..	<i>Eugenia jambos</i>	"	78.21	0.67	0.10	0.41	0.87	19.74	0.016	0.011	1.00	82.5	
* Korukapalli ..	<i>Pithecolobium dulce</i>	"	80.78	2.57	0.31	0.41	..	15.93	0.011	0.036	0.37	76.8	
Lemon ..	<i>Citrus medica</i> var. <i>limonum</i> .	Local	84.97	0.98	0.85	0.26	1.65	11.29	0.070	0.014	2.33	56.7	
Lime ..	<i>Citrus medica</i> var. <i>acidula</i>	"	84.57	1.49	0.97	0.64	1.25	11.08	0.091	0.021	0.29	59.0	
Mango (green) ..	<i>Mangifera indica</i>	Coimbatore	90.03	0.65	0.11	0.37	..	8.84	0.012	0.017	4.48	39.0	
" (type) ..	"	Salem	86.14	0.61	0.07	0.32	1.14	11.69	0.067	0.020	0.25	26.0	

" (Ankola)	"	Bombay	85-94	1-02	0-11	0-46	..	12-47	0-004	0-016	0-45	55-0
Melon, water	<i>Citrullus vulgaris</i>	Coimbatore	95-70	0-12	0-15	0-22	..	3-81	0-001	0-008	0-15	17-1
Orange ..	<i>Citrus aurantium</i>	Local	87-84	0-85	0-32	0-43	..	10-56	0-046	0-022	0-08	48-5
" ..	"	Punjab	91-39	0-85	0-08	0-51	..	7-17	0-023	0-025	0-24	32-4
" (Washington navel)	"	Local	89-76	0-66	0-10	0-31	..	9-17	0-020	0-020	0-18	40-2
" (Jaffa)	"	"	90-78	0-62	0-09	0-31	..	8-20	0-018	0-021	0-24	36-1
Palmyra fruit (tender)	<i>Borassus labellifer</i>	Coimbatore	92-72	0-58	0-02	0-17	..	6-51	0-005	0-021	0-46	28-5
Papaya (ripe)	<i>Carica papaya</i>	Local	89-62	0-47	0-03	0-38	..	9-50	0-013	0-009	0-42	40-2
Peaches	<i>Amigdalus persica</i>	"	90-07	1-52	0-19	0-58	..	7-61	0-006	0-029	1-70	38-4
Pears (country)	<i>Pyrus communis</i>	"	86-92	0-18	0-05	0-27	1-00	11-58	0-006	0-011	0-68	47-5
Pears (English)	<i>P. Achnas</i>	"	85-84	0-94	0-23	0-23	..	12-76	0-005	0-016	0-84	56-9
Pears, 'avocado' or butter fruit.	<i>Persea drymifolia</i>	"	73-55	1-68	22-31	1-05	..	0-91	0-011	0-076	0-71	215-7
Pineapple	<i>Ananas sativa</i>	Local bazaar	86-50	0-57	0-04	0-49	0-40	12-00	0-021	0-007	0-92	50-6
Plantain	<i>Musa paradisiaca</i>	"	73-35	1-06	0-08	0-72	..	24-79	0-011	0-031	0-50	104-1
" 'hill anai-kombu'	"	Local	79-93	1-22	0-06	0-80	..	17-99	0-007	0-025	0-27	77-4
Plum ..	<i>Prunus domestica</i>	"	89-82	0-68	0-22	0-36	..	8-92	0-017	0-018	0-55	40-4
Pomegranate	<i>Punica granatum</i>	Local bazaar	78-90	1-63	0-04	0-67	5-10	14-56	0-009	0-066	0-27	65-1
'Pomelo'	<i>Citrus decumana</i>	Local	88-10	0-63	0-04	0-51	0-60	10-12	0-028	0-027	0-04	43-4
Quince ..	<i>Cydonia vulgaris</i>	"	85-76	0-28	0-01	0-33	1-72	11-87	0-005	0-015	0-44	49-0
Raisins (preserved)	<i>Vitis vinifera</i>	Local bazaar	18-47	1-96	0-18	1-98	..	77-41	0-101	0-075	3-98	319-1
Strawberry (big)	<i>Fragaria grandiflora</i>	Local	87-82	0-71	0-18	0-38	1-14	9-77	0-026	0-026	1-75	43-5
Tomato (ripe)	<i>Lycopersicum esculentum</i>	"	94-52	1-02	0-08	0-48	..	3-90	0-008	0-018	0-08	20-4
" (tree)	<i>Cyphomandra betacea</i>	"	82-72	1-46	0-21	1-12	4-15	10-34	0-013	0-029	0-71	43-3
Wood apple	<i>Feronia elephantum</i>	Coimbatore	69-54	7-31	0-56	1-88	5-16	15-55	0-127	0-107	0-62	96-5
Tamarind (also referred to under condiments).	<i>Tamarindus indicus</i>	Local bazaar	20-86	3-06	0-14	2-86	5-57	67-51	0-172	0-110	10-90	283-5

TABLE Ih.

Condiments, spices, etc.

Name.	Botanical name.	Source.	Moisture per cent.	Protein per cent.	Ether extractives per cent.	Mineral matter per cent.	Fibre per cent.	Carbohydrates per cent.	Calcium (Ca) per cent.	Phosphorus (P) per cent.	Iron (Fe) mg. per cent.	Calorific value per 100 g.	REMARKS.
1	2	3	4	5	6	7	8	9	10	11	12	13	14
'Arisithippili'	<i>Piper elusii</i>	Local bazaar	12.53	13.24	4.66	6.03	5.17	58.37	0.462	0.278	13.54	328.4	
Asafoetida	<i>Ferula narthex</i>	"	16.03	3.95	1.13	6.99	4.12	67.78	0.692	0.045	22.20	297.1	
Cardamom	<i>Elettaria cardamomum</i>	"	19.95	10.21	2.18	5.37	20.10	42.19	0.128	0.163	5.00	229.2	
Chillies (green)	<i>Capiscum annuum</i>	"	82.60	2.87	0.59	1.04	6.73	6.17	0.029	0.079	1.31	41.5	
" (dry)	"	"	10.02	15.88	6.24	6.10	30.15	31.51	0.163	0.371	2.25	245.7	
Cloves	<i>Eugenia caryophyllata</i>	"	23.29	5.24	8.90	5.20	9.54	47.83	0.739	0.097	4.90	292.4	
Coriander seeds	<i>Coriandrum sativum</i>	"	11.15	14.08	1.04	4.39	32.58	36.76	0.630	0.366	17.94	212.7	
Cumin	<i>Cuminum cyminum</i>	"	11.94	18.71	0.58	5.84	11.98	50.95	1.083	0.487	31.00	283.9	
fenugreek seeds	<i>Trigonella fenugracum</i>	"	13.70	26.19	5.77	3.00	7.16	44.18	0.157	0.365	14.10	333.4	
Garlic	<i>Allium sativum</i>	"	62.79	6.31	0.13	1.02	0.80	28.95	0.025	0.305	1.31	142.2	
Ginger	<i>Zingiber officinale</i>	"	80.86	2.33	0.94	1.19	2.42	12.26	0.016	0.063	2.57	66.8	
'Kandanthippili'	<i>Piper roxburghii</i>	"	12.24	6.44	2.32	4.77	8.51	65.72	1.225	0.188	62.10	309.5	Roots.
Mace	<i>Myristica fragrans</i>	"	15.92	6.51	24.36	1.58	3.76	47.87	0.181	0.103	12.64	436.8	
Mustard	<i>Brassica juncea</i>	"	8.46	22.04	39.64	4.19	1.80	23.87	0.488	0.704	17.88	540.4	
(also referred to under oil-seeds).													
Nutmeg	<i>Myristica fragrans</i>	"	14.32	7.49	36.44	1.72	11.59	28.44	0.122	0.235	4.57	471.7	
Onum	<i>Carum copiticum</i>	"	8.92	15.41	18.08	7.09	11.87	38.63	1.422	0.297	14.62	378.9	
Onion (big)	<i>Allium cepa</i>	"	86.76	1.22	0.04	0.36	..	11.62	0.176	0.046	0.66	51.7	
" (small)	"	"	84.33	1.80	0.08	0.55	..	13.24	0.040	0.058	1.18	60.9	
Pepper (dry)	<i>Piper nigrum</i>	"	12.86	11.53	6.79	4.40	14.92	49.50	0.460	0.199	16.80	305.2	
Tamarind (also referred to under fruits).	<i>Tamarindus indicus</i>	"	20.86	3.06	0.14	2.86	5.57	67.51	0.172	0.110	10.90	283.5	Pulp only.
Turmeric	<i>Curcuma longa</i>	"	13.08	6.33	5.10	3.51	2.60	69.38	0.146	0.284	18.60	348.7	



TABLE II.  
*Flesh foods.*

Name.	2	3	4	5	6	7	8	9	10	11	12
	Moisture per cent.	Protein per cent.	Ether extractives per cent.	Mineral matter per cent.	Fibre per cent.	Carbohydrates per cent.	Calcium (Ca) per cent.	Phosphorus (P) per cent.	Iron (Fe) mg. per cent.	Calorific value per 100 g.	REMARKS.
1											
Beef (muscle)	74.33	22.61	2.58	0.99	..	..	0.004	0.190	0.77	113.7	Varieties of fish commonly con- sumed on the West Coast.
Crab ( " )	83.49	8.87	1.07	3.20	..	3.37	1.370	0.147	21.20	58.6	
Egg (duck) ..	71.01	13.54	13.72	1.03	..	0.70	0.073	0.261	2.97	180.4	
" (fowl) ..	73.65	13.33	13.32	0.95	..	..	0.056	0.271	2.07	173.2	
Fish, Mangalore (muscle)	78.38	22.60	0.06	0.83	..	..	0.020	0.185	0.83	90.9	
" " ( " )	77.89	21.49	1.63	2.03	..	..	0.055	0.413	2.28	100.6	
" Vajra ( " )	79.41	19.92	1.51	1.38	..	..	0.044	0.379	0.72	93.3	
Liver (sheep)	70.37	19.29	7.49	1.45	..	1.40	0.006	0.378	6.30	150.2	
Mutton (muscle)	71.46	18.54	13.30	1.26	..	..	0.147	0.151	2.52	193.9	
Pork ( " )	77.41	18.66	4.40	1.01	..	..	0.034	0.198	2.27	114.2	
Prawn ( " )	77.94	20.84	0.28	1.42	..	..	0.088	0.244	0.79	85.9	

TABLE Ij.  
*Miscellaneous foodstuffs.*

Name.	Moisture per cent.		Protein per cent.		Ether extractives per cent.		Mineral matter per cent.		Fibre per cent.		Carbohydrates per cent.		Calcium (Ca) per cent.		Phosphorus (P) per cent.		Iron (Fe) mg. per cent.		Calorie value per 100 g.		REMARKS.
	1	2	3	4	5	6	7	8	9	10	11	12									
Jaggery or 'ghur' ..		3.93	0.36	0.06	0.62	..	95.03	0.075	0.038	11.40	382.1										
Curds ..		90.31	2.86	2.94	0.59	..	5.30	0.120	0.088	0.31	59.1										
Butter-milk ..		97.54	0.84	1.08	0.14	..	1.40	0.030	0.030	0.78	18.7										
Betel leaves ( <i>Piper betle</i> )		85.41	3.07	0.80	2.25	2.33	6.14	0.227	0.044	5.74	44.0										
'Perandai' ( <i>Vitis quadrangularis</i> ).		87.42	1.21	0.34	2.01	1.83	7.19	0.651	0.050	2.12	36.7										
'Pappads' ..		20.32	18.76	0.33	8.17	..	52.42	0.080	0.298	17.24	287.7										Black gram pre- paration.
Sweet toddy ..		84.72	0.10	0.17	0.66	..	14.35	0.149	0.011	0.26	59.3										
Skim-milk powder ..		4.10	83.04	0.09	6.83	..	50.94	1.370	0.995	1.43	356.7										

Over 200 foodstuffs were analysed by the methods mentioned above. The fractional analysis of the ash, viz., calcium, phosphorus, and iron, was done in duplicate and the results, given in columns 10, 11, and 12, represent the mean of the two results. The results, expressed as a percentage of the original sample, are set forth in the tables (Table I *a* to *j*); Table II summarizes roughly the results for each group of foodstuffs:—

TABLE II.

*Showing the average chemical composition of each group of foodstuffs.*

Group of foodstuffs.	Moisture per cent.	Protein per cent.	Ether extractives per cent.	Total mineral matter per cent.	Carbohydrates per cent.	Calcium (Ca) per cent.	Phosphorus (P) per cent.	Iron (Fe) mg. per cent.	Calorific value per 100 g.
Cereals .. ..	14.53	8.53	1.54	1.78	71.91	0.052	0.224	4.66	335.7
Pulses .. ..	11.48	24.47	3.39	3.08	55.58	0.135	0.387	6.92	350.7
Leafy vegetables ..	80.81	4.89	0.85	2.16	10.21	0.334	0.080	11.45	68.1
Root and tuber vegetables.	77.85	1.31	0.14	0.95	19.57	0.054	0.042	0.90	84.8
Other vegetables ..	85.17	2.12	0.25	1.00	9.98	0.058	0.053	2.38	50.7
Nuts and oil-seeds ..	8.96	20.08	46.54	2.69	19.07	0.273	0.448	6.15	575.5
Fruits .. ..	79.63	1.28	0.72	0.65	16.50	0.027	0.032	1.26	77.6
Condiments, spices, etc.	29.64	9.56	7.86	3.68	40.13	0.407	0.232	12.51	269.6
Flesh foods ..	75.94	18.15	5.40	1.41	0.50	0.172	0.252	3.87	123.2

### DISCUSSION.

The cereals are rich in carbohydrates and fairly rich in proteins, their protein content ranging from 7 to 12 per cent. Their content of fat and calcium is small in comparison with that of certain other kinds of foodstuffs; iron and phosphorus are present in relatively greater amounts. They contain, as a rule, more phosphorus than calcium, just the reverse of green leafy vegetables, which suggests one of the advantages of supplementing a largely cereal diet by vegetables.

Pulses are good sources of protein, containing on an average 24.47 per cent. They are somewhat richer than cereals as regards most chemical constituents, and on the average more than twice as rich in proteins.

The vegetables are, in general, poor sources of the 'proximate principles', but are fairly good sources of the mineral salts. Most of them contain fair amounts of fibre and therefore supply roughage. The leafy vegetables are exceptionally good sources of iron.

The nuts and oil-seeds are naturally rich in fats; they contain in addition fair amounts of protein, calcium, phosphorus, and iron.

The fruits are poor sources of the 'proximate principles' and mineral salts. Their chief dietary value is probably their richness in ascorbic acid or vitamin C.

The condiments and spices are mostly good sources of iron; a few of them are also rich in calcium and phosphorus.

In general, the iron content of the foodstuffs analysed was found to exceed the iron-content figures given in standard textbooks. In this respect our analyses correspond with those carried out by Rosedale (*loc. cit.*) on Malayan foods.

Special mention may be made of a few common and inexpensive foodstuffs, grown and available in abundance in India, which appear, on the basis of chemical analysis, to possess high nutritive value. Thus, amaranth leaves and drumstick leaves, despite their high moisture content—about 80 per cent—are rich in proteins, calcium, phosphorus, and iron. They are very cheap, especially in the rural parts of India. Again, soya bean, a foodstuff that is receiving wide attention in India and elsewhere, is unique among the pulses in containing over 40 per cent of protein. It is also rich in fats and in the three minerals. A pound of it costs about an anna, and the cost per unit of money, of protein and fat, in this form is relatively small.

It must be emphasized that the chemical analysis of foodstuffs, as described in this paper, requires supplementing in certain directions. While it is valuable to know the percentage of proteins a foodstuff contains, it is also necessary to know the biological value of the proteins; similarly the *availability* of mineral salts contained in a food may be as important as their concentration. Further, the analyses were carried out on foods in the state in which they are bought and not in the state in which they are consumed. Investigations are being carried out in the laboratories to throw light on all these points, and to complete the survey in its various aspects.

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## THE STATE OF NUTRITION OF SCHOOL CHILDREN IN SOUTH INDIA.

### Part II.

#### DIET AND DEFICIENCY DISEASE IN RESIDENTIAL HOSTELS.

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IN a previous paper published from these Laboratories (Aykroyd and Rajagopal, 1936) the results of the examination of some 2,000 day-school children in three South Indian towns were given. The objects of the present investigation, in which we were concerned with children in residential hostels, may be described as follows: we wished (*a*) to collect information about institutional diets in South India, (*b*) to study the 'state of nutrition', assessed by various criteria, of children whose diet was known with reasonable accuracy, and (*c*) to compare their condition with that of children in the general population. With regard to (*b*), special attention was given to the incidence of certain symptoms of food deficiency disease—phrynoderma, angular stomatitis, and Bitot's spots—to which reference was made in previous papers (Aykroyd and Krishnan, 1936*b* and *c*; Aykroyd and Rajagopal, *loc. cit.*). We hoped that by observing the incidence of these symptoms in relation to diets of known composition it might be possible to discover the precise deficiencies which give rise to them; another possibility was the discovery of how much vitamin A and carotene in the diet are needed to prevent Bitot's

spots (xerophthalmia). It will be shown how far the original objects of the investigation were attained.

#### METHOD OF INVESTIGATION.

Residential hostels for children abound in South Indian towns, their usual purpose being to provide a home for country children studying at school. The majority of hostels visited by us are supported by Christian Missions of various denominations; a few are run by Hindu Missions or by the Government. Generally speaking, mission hostels have to feed and lodge children at a very low level of expenditure; government hostels are financed on a slightly more generous scale. The children in Christian Mission hostels belong, for the most part, to the 'depressed classes' and come from rural districts. Children usually remain in the hostels for a number of years, returning home for brief holidays three times a year.

In all we visited 30 hostels. All except two of these were situated in various parts of the Madras Presidency; the two exceptions were located in Mysore. In the final records we have eliminated six hostels, owing, in most cases, either to uncertainty regarding the accuracy of food records obtained or to some other irregularity or drawback (e.g., frequent absences of children which made it difficult to calculate food intake per consumption unit). The investigation, as here presented, includes 24 hostels, housing 719 boys and 955 girls, of ages ranging from 4 to 20 years. The majority fell in the age groups 9 to 17. In each hostel we weighed and measured the children and examined them for phrynoderma, angular stomatitis, and Bitot's spots. Standards of examination were those previously described (Aykroyd and Rajagopal, *loc. cit.*). Particulars about diet were obtained as follows: a single 'typical' month previous to the investigation was selected, and records of expenditure on food during that month were obtained; we found that almost invariably a monthly supply of the main items in the diet was bought at the beginning of each month, leaving little over at the end. Further questions were put to the housekeeper or superintendent to fill in gaps; it was always necessary, for example, to inquire about the supply of fresh vegetables, usually purchased two or three times a week in the local market. It may be objected that any adequate picture of diet throughout the year cannot be obtained from the food intake of a single month, which might be any month of the twelve. But we found that there is very little variation in diet throughout the year in hostels of the type visited. Expenditure on food in most cases was at the level of Rs. 3 to Rs. 4 per head per month, and at this level little variation is possible in the general composition of the diet (e.g., in the quantity of cereal relative to that of other foods). In most cases we were told that the diet schedules had remained the same for some years. We are satisfied that in each case the diet supplied throughout the year *resembled closely* the diet supplied during the month chosen for investigation.

Table I gives the content of the various diets as regards calories, proximate principles, calcium, phosphorus, and iron, with the percentage of total calories derived from cereals. The percentage incidence of phrynoderma, angular stomatitis, and Bitot's spots in the various groups is recorded. In Table II, the foodstuff composition of a few of the diets is given; these may be regarded as typical of the remainder.

TABLE I.  
*Diet and percentage incidence of deficiency disease symptoms in boys' hostels.*

Hostel No. :—	1	2	3	4	5	6	7	8	9	10	11	12	13	Mean.
<i>Number of boys :—</i>	63	32	50	61	51	53	60	21	185	30	113	..	..	65
Protein (g.) ..	96	56	55	79	72	63	72	78	47	79	80	..	..	71
Fat (g.) ..	56	10	10	28	54	33	21	23	15	51	25	..	..	30
Carbohydrates (g.) ..	533	410	458	559	496	459	501	570	404	482	451	..	..	484
Calories ..	3,033	1,945	2,141	2,793	2,763	2,382	2,482	2,713	1,961	2,728	2,352	..	..	2,481
Calcium (g.) ..	0.35	0.16	0.18	0.27	0.79	0.24	1.22	0.35	0.20	0.51	0.28	..	..	0.42
Phosphorus (g.) ..	0.94	0.99	0.97	2.40	1.30	1.04	1.72	2.08	1.05	1.42	1.70	..	..	1.42
Iron (mg.) ..	23.70	19.30	17.40	52.10	31.70	26.10	40.60	22.60	22.00	34.40	42.50	..	..	30.20
Vitamin A ( $\gamma$ ) ..	61	25	21	45	232	3	36	14	2	2	2	..	..	..
Carotene ( $\gamma$ ) ..	1,815	617	688	1,401	1,830	1,225	2,100	209	515	925	1,138	..	..	..
Percentage calories derived from cereals.	64	86	88	77	64	75	82	83	85	56	82	..	..	..
Percentage incidence of angular stomatitis.	32	50	51	34	4	30	0	25	7	0	13	..	..	..
Percentage incidence of Bitot's spots.	6	3	16	23	4	4	5	0	6	8	7	..	..	..
Percentage incidence of pityrioderma.	11	34	22	53	0	13	9	0	6	4	4	..	..	..

TABLE I—*concd.**Diet and percentage incidence of deficiency disease symptoms in girls' hostels.*

Hostel No.:—	1	2	3	4	5	6	7	8	9	10	11	12	13	Mean.
Number of girls:—	50	58	36	87	66	54	88	69	100	167	39	81	60	74
Protein (g.) ..	63	62	64	52	59	63	87	58	59	54	87	60	67	64
Fat (g.) ..	61	23	13	10	12	25	34	30	16	20	34	13	39	25
Carbohydrates (g.) ..	422	429	475	412	432	386	448	361	411	412	532	503	444	436
Calories ..	2,418	2,167	2,273	1,912	2,073	2,011	2,460	1,948	2,017	2,024	2,788	2,370	2,496	2,221
Calcium (g.) ..	0.72	0.76	0.41	0.54	1.53	0.63	0.80	0.66	0.52	0.31	0.78	0.85	0.54	0.70
Phosphorus (g.) ..	1.25	1.25	1.19	1.14	1.52	1.24	2.19	1.30	1.09	1.09	1.42	1.28	1.34	1.35
Iron (mg.) ..	29.20	33.10	24.50	25.80	35.30	18.70	58.30	35.80	24.70	22.70	22.70	27.60	32.70	30.10
Vitamin A ( $\gamma$ ) ..	294	161	2	12	2	26	1	25	35	243	260	2,133	906	..
Carotene ( $\gamma$ ) ..	2,733	1,857	1,235	1,141	2,021	2,123	2,702	1,730	1,721	1,354	4,192	985	1,795	..
Percentage calories derived from cereals.	60	70	84	90	88	77	78	82	75	80	66	92	71	..
Percentage incidence of angular stomatitis.	0	0	71	8	0	0	0	3	0	1	0	9	14	..
Percentage incidence of Bitot's spots.	2	0	3	5	11	16	6	1	1	10	0	3	4	..
Percentage incidence of phrynoderma.	4	0	34	33	27	46	6	12	25	5	5	4	0	..



TABLE II.

Foodstuff composition of various hostel diets.  
(Oz. per consumption unit per day.)

Boys' HOSTEL.			Girls' HOSTEL.		
No. 6	No. 7.	No. 8	No. 3.	No. 5	No. 6
Milled parboiled rice.	180	Home pounded par-boiled rice	175	Milled parboiled rice.	Milled parboiled rice.
Dhal arhar ( <i>Cajanus indicus</i> )	18	Whole wheat	15	Ragi	Ragi
Bengal gram ( <i>Cicer arietinum</i> )	01	Ragi	15	Dhal arhar	Cholam ( <i>Sorghum andropogon</i> ).
Tamarind	06	Dhal arhar	03	Bengal gram	Dhal arhar
Coriander seeds	01	Bengal gram	03	Coriander seeds	Black gram
Onions	03	Green gram ( <i>Pisaceo lus mungo</i> )	05	Brinjal	Brinjal
Brinjal ( <i>Solanum melongena</i> )	06	Horse gram ( <i>Dolichos biflorus</i> )	02	Onions	Onions
Pumpkin	12	Tamarind	01	Potato	Pumpkin
Potato	06	Coriander seeds	03	Plantains	Cucumber
Amaranth ( <i>Amaranthus gangeticus</i> )	16	Onions	01	Meat	Green beans
Plantains	02	Bitter gourd ( <i>Momordica charantia</i> ).	01	Gingelly oil	Potato
Oranges	02	Ridge gourd	01		Drumstick
Meat	02	Cucumber	01		Amaranth
Coco-nut	03	Pumpkin	01		Plantains
Gingelly oil	03	Lady's fingers ( <i>Hibiscus esculentus</i> ).	01		Nutton
		Green beans	01		Eggs
		Spinach	01		Coco-nut
		Fish	01		Gingelly oil
		Whole milk	02		Jaggery
		Coco-nut	02		
		Coco nut oil	02		
		Jaggery	02		

Intake was calculated per consumption unit ('man value') per day, the earlier International scale of family co-efficients (League of Nations Health Organization, 1932) being employed.

#### COMPOSITION OF THE DIETS.

The diets as shown in Tables I and II are very typical of those supplied in children's hostels throughout a great part of India, and closely resemble those consumed by the general population. The most common cereal staple used is parboiled milled rice, but millet (usually ragi) is in many instances provided as part of the grain ration. The chief pulse is dhal arhar. Green leafy and other vegetables, and fruit, are provided in relatively small quantities, and as a general rule milk is absent from the diet, or given in very small quantities a few times a week as an addition to tea. A meat meal once a week is often supplied to relieve monotony, and is greatly relished by the children. The chief cereal supplied in hostels usually corresponds to the chief cereal grown in the area in which the hostel is situated.

Average calorie intake in the boys' hostels approximates to the figure which we regard as representing minimum requirements per average man or consumption unit in South India. We were usually informed by hostel superintendents that the children were much more liberally fed in the hostels than in their own homes; entrants are often severely under-nourished and put on a good deal of weight during the months after admission; weight is often lost during the holidays. While it appears that in a number of the hostels the food supply tended to be insufficient in quantity, there can be little doubt that in general the statement of superintendents regarding under-nourishment on admission and subsequent improvement is correct. Diet surveys of poor families of a type similar to those from which the hostel children are drawn would probably reveal a lower calorie intake per consumption unit, and hostel intake is a *better* guide to requirements than intake in the general population. Obviously, however, it is not a completely satisfactory guide, since such institutions are for the most part run at a minimum level of expenditure, and may fail to supply sufficient food fully to satisfy the children's appetites.

Average energy intake per consumption unit was lower in the girls' hostels than in the boys'. Actually we gained the impression that catering arrangements in the girls' hostels were more efficient, the reason being that missionaries and superintendents in charge of girls' hostels are of the female sex. On the whole, the girls were a better nourished group. Theoretically, if food consumption is just adequate in two groups, male and female respectively, calories per consumption unit should work out to the same figure, allowance being made in the scale of consumption co-efficients for lower female requirements. If, in such circumstances, they do not, the scale of co-efficients must be at fault. We are inclined to think that this is the case in the present instance. In the International scale, female consumption is reckoned as 0.8 of male consumption in the age groups over 14, and probably this factor is too high. Mason and Benedict (1931) have shown that basal metabolism of a group of South Indian women was 17 per cent below American standards, while according to Rahman (1936) that of a group of young male students

in Hyderabad was only about 7 per cent below the same standards. Adolescent Indian girls, compared with their brothers, are placid and do not play energetic games. Probably, therefore, a lower fraction of male requirements should be assigned to females over 14. 0.7 may be suggested. If calorie intake in the girls' hostels were worked out on this basis, it would approximate more closely to that calculated in the boys' hostels. The lower calorie intake in the girls' hostels does not justify the assumption that the food of the girls was less adequate in quantity than that of the boys.

Average protein intake per consumption unit was fairly high, above that observed in village-diet inquiries (Aykroyd and Krishnan, 1937). Very little protein of animal origin was consumed. Fat consumption was low and animal fat was lacking. Differences in protein and fat intake in the various hostels were to a considerable extent dependent on the relative quantities of rice and millet consumed. Average calcium intake in the boys' hostels was low, the highest intake being observed in institutions in which ragi formed a considerable part of the diet. In the girls' hostels, ragi was more commonly given, and calcium intake was relatively greater. Phosphorus and iron appear to be present in sufficient amounts in all the diets. The question of vitamin intake will be discussed later in considering the relation between deficiency disease and the quality of the diets.

#### COST OF DIETS.

In the majority of hostels, cost of food was between Rs. 3 and Rs. 4 per child per month. In a few cases this level of expenditure was exceeded.

#### HEIGHT AND WEIGHT.

In the majority of hostels we weighed and measured the children ourselves; in some cases, however, we have used records obtained by the superintendent a short time previous to our visit, taking the precaution of checking the accuracy of the scales used against a balance of known accuracy. Originally we intended to compare height and weight in the various hostels as an index of the nutritive value of the diets. This project was, however, abandoned because of the statistical difficulties involved in comparing small numbers belonging to various age groups and also because of lack of knowledge about variation in physique in the parts of the country covered by the inquiry. It is, however, worth while to compare composite height-weight-age averages, based on children in all the hostels, with certain curves based on children of the general population.

Table III gives the average height and weight of hostel boys and girls in the age groups 10 to 17.

In Figs. 1, 2, 3, and 4 these height-weight-age averages are compared with those of day-school children between the ages of 10 and 15 (boys) and 8 and 15 (girls). The day-school boys' measurements were obtained in a previous investigation (Aykroyd and Rajagopal, *loc. cit.*), the children concerned belonging to three South Indian towns. The day-school girls' averages are those published in the Annual Report of the Health Department, Corporation of Madras, for the year 1934; no adequate female height-weight data, relating to the general population,

has as yet been collected by us. The day-school and the hostel groups are roughly comparable as regards the class of children concerned.

TABLE III.

*Average height and weight of boys and girls in residential institutions in South India.*

Age.	Boys.			Girls.		
	Number weighed and measured.	Height (inches).	Weight (pounds).	Number weighed and measured.	Height (inches).	Weight (pounds).
10	31	51.17	55.02	38	50.23	53.68
11	65	52.06	58.00	82	52.09	57.85
12	98	53.45	60.66	86	53.41	64.88
13	94	56.43	70.09	108	55.82	73.07
14	102	58.83	81.06	75	57.28	80.49
15	73	59.46	81.99	89	58.16	87.95
16	69	61.82	95.25	64	58.40	88.11
17	81	63.35	100.46	46	58.57	89.50

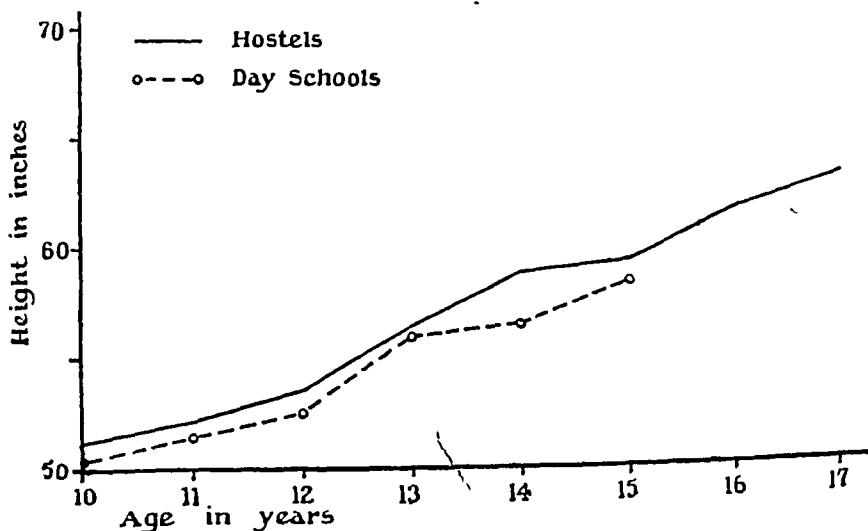


Fig. 1. Height-age averages of boys in hostels and day schools.

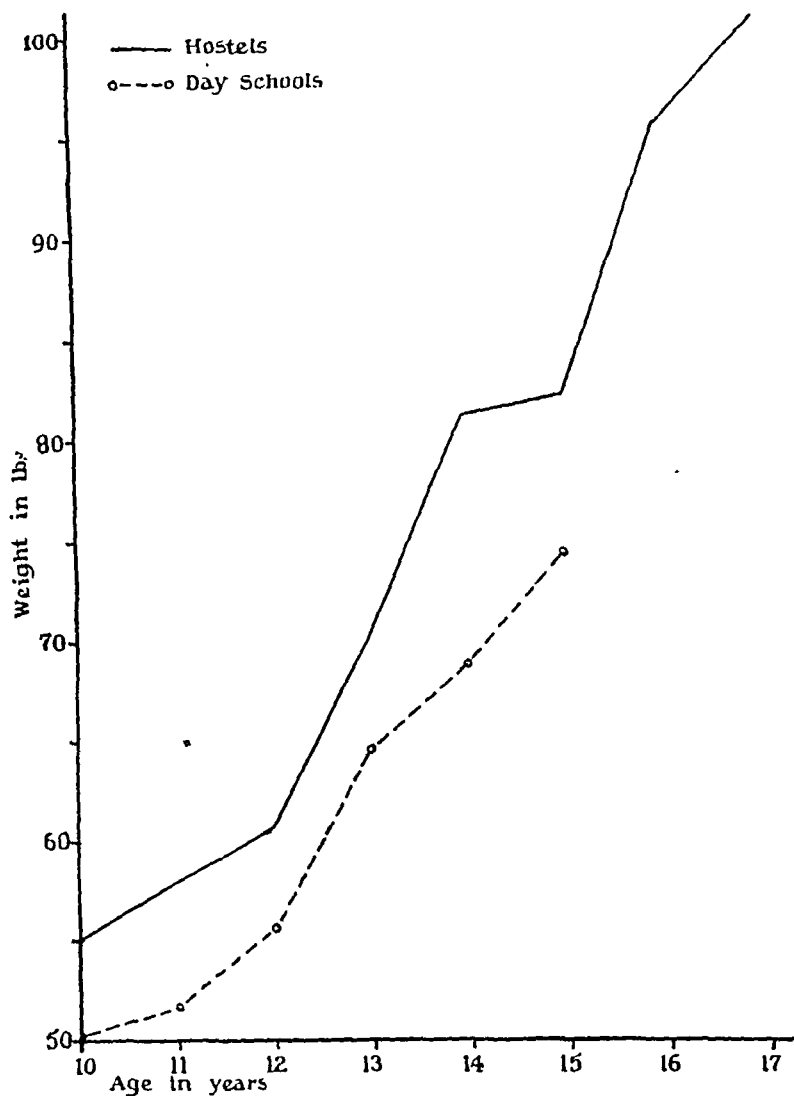


Fig. 2. Weight-age averages of boys in hostels and day schools.

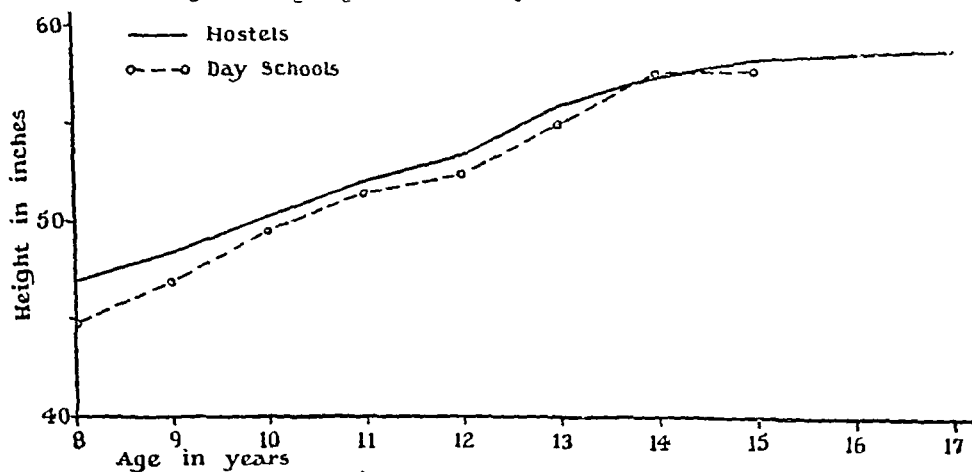


Fig. 3. Height-age averages of girls in hostels and day schools.

Figs. 1 to 4 show that both the height and weight averages of the hostel children are above those of day-school children, the advantage being greater as regards

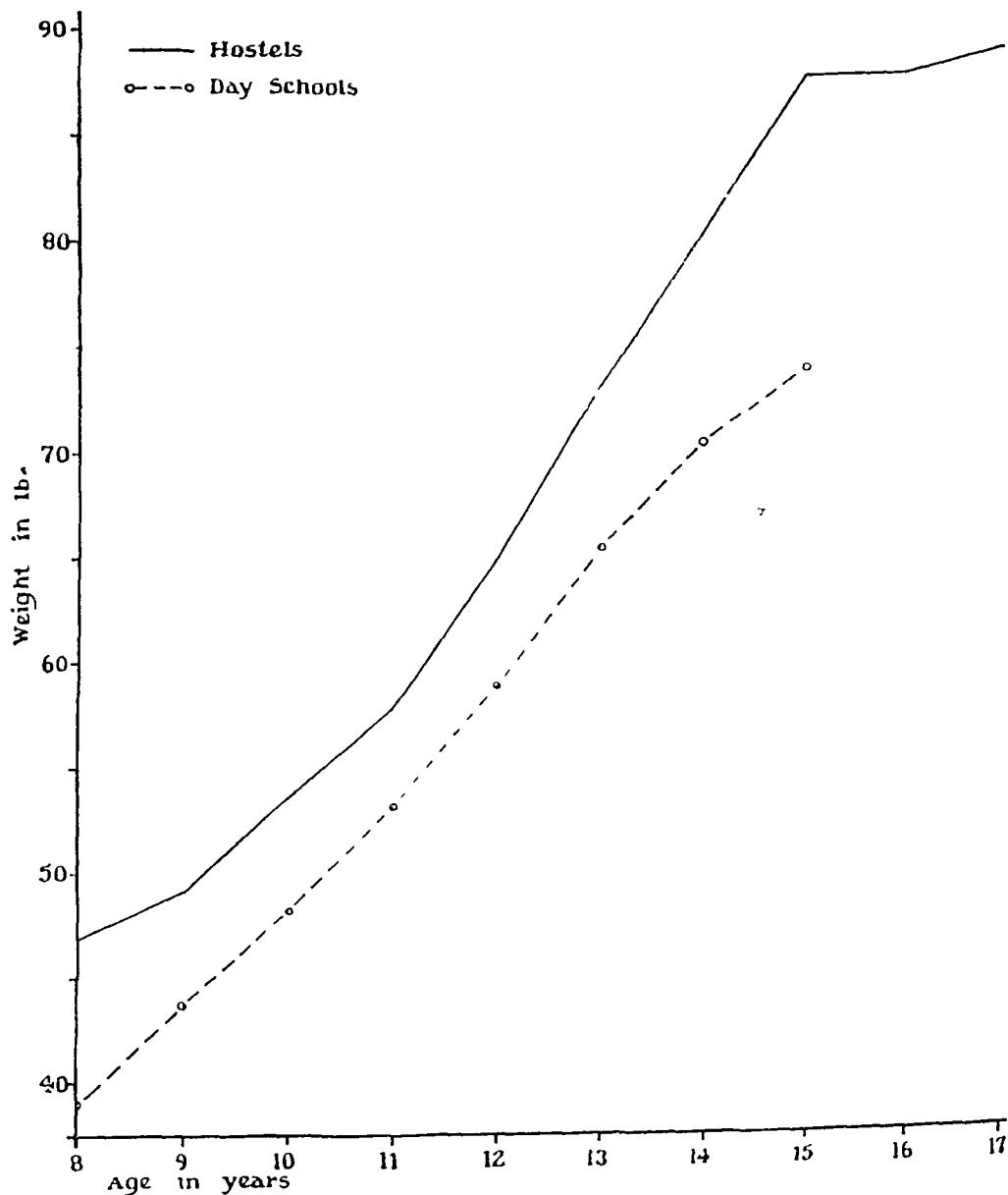


Fig. 4. Weight-age averages of girls in hostels and day schools.

weight. To demonstrate the latter advantage more clearly, curves (Figs. 5 and 6) of average weight on average height in the age groups 10 to 15 of hostel and

day-school children have been plotted. It will be observed that at every age the weight for height of the hostel children is greater than that of the day-school children. This difference is, in our opinion, an expression of the better 'state of nutrition' of the children living in institutions. Their diet may in many instances be poor in quality, but at least they regularly consume three meals per day,

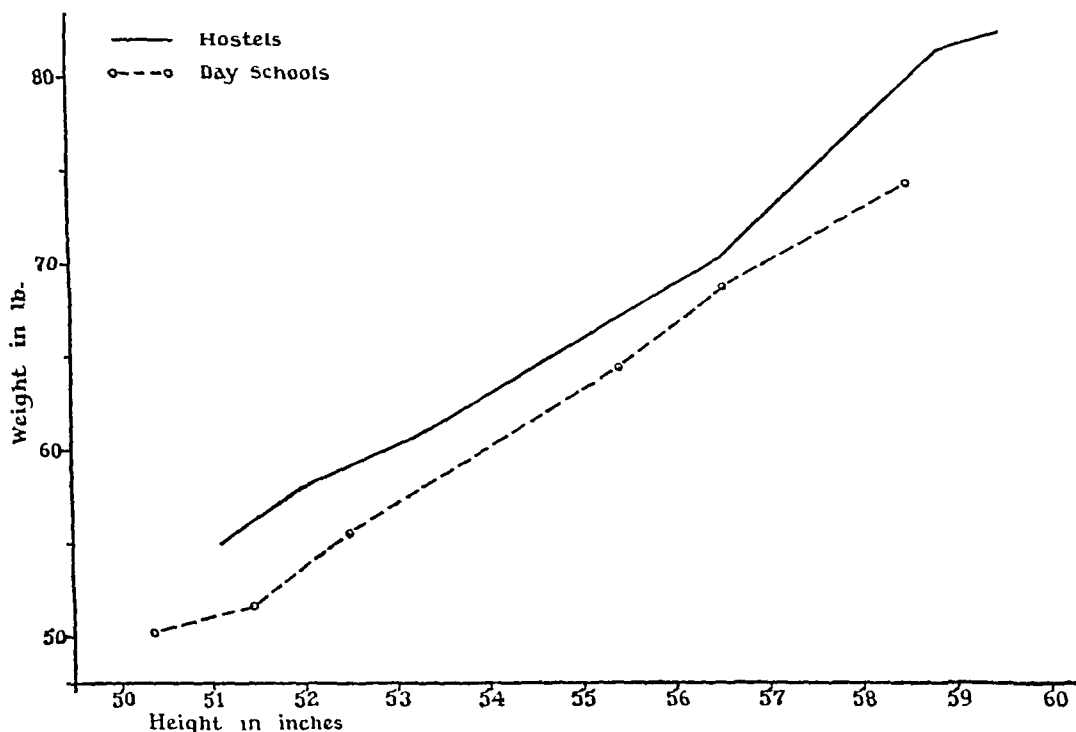


Fig. 5. Weight for height of hostel and day-school boys compared.

while children of the poorer classes in the general population may be less fortunate in this respect. The fact that day-school children are on the average thinner than children fed on diets costing Rs. 3 to Rs. 4 per month throws a light on the diet and economic conditions of the former.

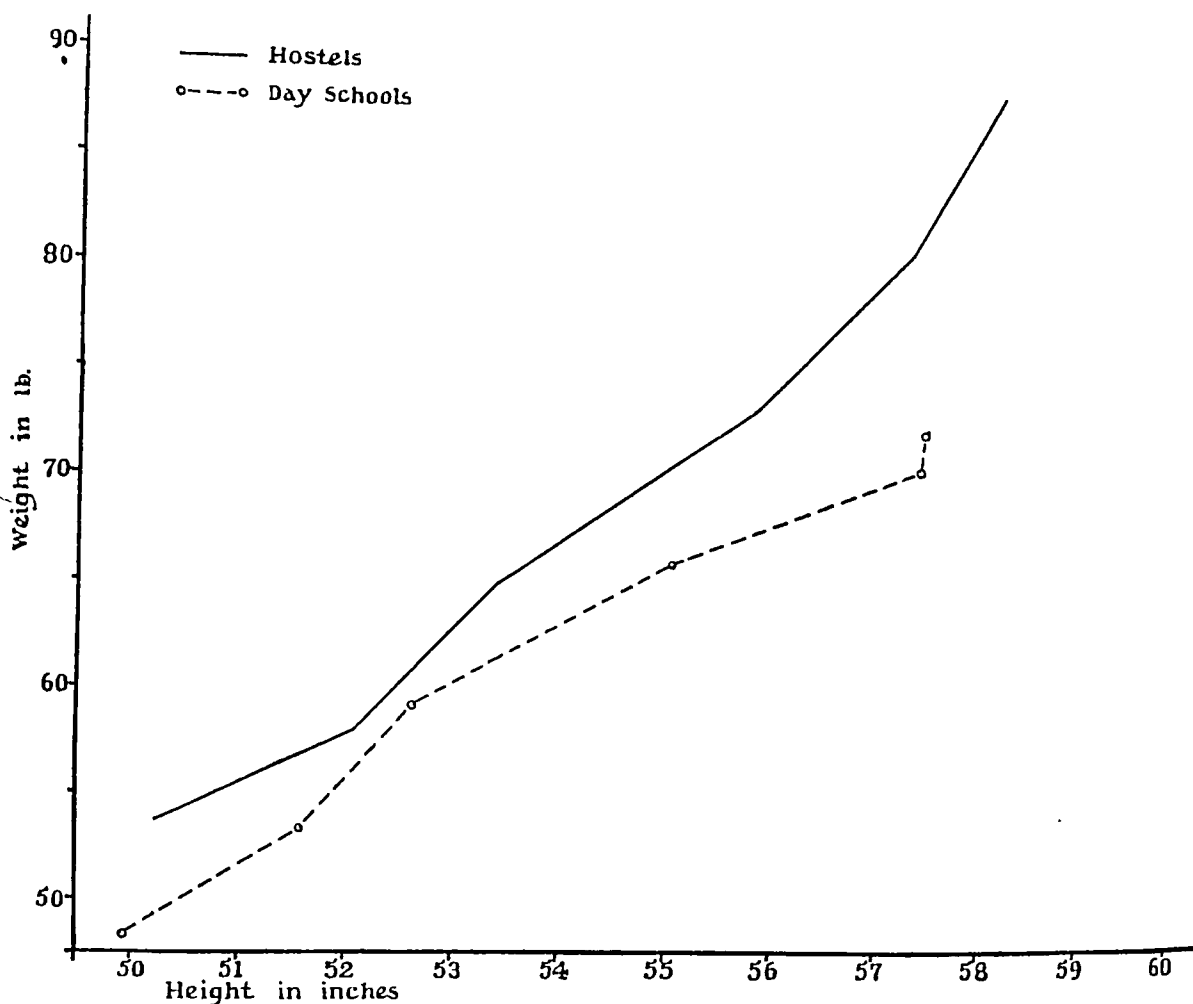


Fig. 6. Weight for height of hostel and day-school girls compared.

#### THE INCIDENCE OF SYMPTOMS OF FOOD DEFICIENCY DISEASE.

In a previous investigation (Aykroyd and Rajagopal, *loc. cit.*) some 2,000 day-school children in three South Indian towns were examined for angular stomatitis, phrynoderma, and Bitot's spots. It was observed that these symptoms were frequently present. The methods of examination followed here were similar to those previously described. We found that in general these conditions are common in children living in institutions. In recording the percentage incidence, we disregarded children who had been resident for less than 6 months.

*Angular stomatitis.*—This condition has been fully described in a previous paper (Aykroyd and Krishnan, 1936c). It was pointed out that the characteristic fissures at the angles of the mouth are usually found in association with lesions of the tongue, and that for the systematic examination of large numbers, angular stomatitis is convenient. Both glossitis and angular stomatitis disappear simultaneously on treatment with dried yeast, unheated or autoclaved, and skim-milk. Deficiency of some part of the vitamin-B<sub>2</sub> complex appears to be the causative factor.



As the present investigation proceeded we became aware of a relationship between the nature of the diet and the incidence of angular stomatitis. The condition was most prevalent in hostels in which the bulk of the diet was composed of milled rice, raw and parboiled, and the supply of milk and vegetables was small. In a few hostels, a relatively high consumption of rice was not associated with a high incidence of stomatitis; in these the rice was supplemented by reasonable quantities of milk, vegetables, or (in one case) liver. Where the staple cereal was millet, or where rice and millet were both provided, the latter in fair quantities, angular stomatitis was relatively infrequent. In practice we found that, having examined the children and noted the incidence of stomatitis, we could 'predict' with fair accuracy the nature of the diet. Conversely, we were prepared to wager that cases of stomatitis would be found in any hostel in which an insufficiently supplemented rice diet was supplied.

In Table IV, an attempt is made to demonstrate the relation between stomatitis and the composition of the diet. Hostels are listed in descending order as regards the incidence of stomatitis. The percentage of total calories obtained from rice and the milk intake are given in adjacent columns. Vegetable intake is roughly indicated as above or below 5 oz.

TABLE IV. .

*Diet and angular stomatitis.*

Number of hostel.	Percentage incidence of angular stomatitis.	Percentage of total calories from milled rice.	Milk intake per consumption unit per day (oz.).	Vegetable intake (above or below 5 oz. per consumption unit per day).	REMARKS.
G 3 ..	71	76	None	Below	0.3 oz. liver per consumption unit per day.
B 3 ..	51	86	Butter-milk, 1.3	Above	
B 2 ..	50	78	None	Below	
B 4 ..	34	77	Butter-milk, 1.0	Below	
B 1 ..	32	62	Whole milk, 1.3	Below	
B 6 ..	30	75	None	Above	
B 8 ..	25	83	Butter-milk, 3.8	Below	
G 13 ..	14	49	None	Above	
B 11 ..	13	42	None	Below	
G 12 ..	9	57	None	Below	
					0.75 oz. liver per consumption unit per day.

TABLE IV—*concl'd.*

Number of hostel.	Percentage incidence of angular stomatitis.	Percentage of total calories from milled rice.	Milk intake per consumption unit per day (oz.).	Vegetable intake (above or below 5 oz. per consumption unit per day).	REMARKS.
G 4 ..	8	60	Curds, 0.6	Below	Very high pulse intake.
B 9 ..	7	69	None	Below	
B 5 ..	4	55	Whole milk, 4.6	Above	
G 8 ..	3	23	Whole milk, 1.2	Below	
G 10 ..	1	69	Whole milk, 0.3	Above	
B 7 ..	0	43	Whole milk, 0.6	Above	
B 10 ..	0	56	Butter-milk, 0.2	Below	
G 1 ..	0	50	Whole milk, 6.4	Above	
G 2 ..	0	62	Whole milk, 5.8	Above	
G 5 ..	0	31	None	Below	
G 6 ..	0	33	None	Below	
G 7 ..	0	7	None	Above	
G 9 ..	0	64	Whole milk, 1.8	Above	
G 11 ..	0	20	Whole milk, 6.7	Above	

In the first seven hostels listed, in which the greatest incidence of stomatitis was found, the percentage intake of rice was high, while milk and vegetable consumption was low. It must be observed that 'butter-milk' in India is often a well-watered product of dubious nutritive value. In the group of hostels in which stomatitis was infrequent or absent, we found in general either a low intake of rice, one of the millets being the staple cereal, or a relatively higher intake of rice combined with a fair intake of milk, vegetables, or other nutritious foods. Attention may be drawn to certain hostels. We have previously demonstrated (Aykroyd and Krishnan, 1936c) the curative effect of skim-milk on stomatitis and the value of liver has been observed by Landor and Pallister (1935); according to these workers, stomatitis in prisoners was cured by giving 8.0 oz. of fresh liver daily. In B 10, G 2, and G 9 there was no stomatitis in spite of a relatively high rice intake; in these hostels there was a fair provision of milk or vegetables, or, in one instance, the consumption of pulses was exceptionally high. In G 5 and G 6 there was no stomatitis, in spite of the absence of milk and a low intake of vegetables. The chief cereal consumed in these hostels was millet (ragi in the former; ragi and cholam in the latter), which formed the bulk of the diet. In G 7 also the principal cereal was millet.

It is not to be expected that all the hostels should fit exactly into the scheme; apart from inevitable inaccuracies in the collection of food consumption data, there are many variables which have not been considered, e.g., intake of pulses and other foods, the varying composition of the vegetable ration as regards leafy and other vegetables, and the varying age composition of the hostel groups. For example, in B 9 there was a relatively high rice intake, no milk, and a poor vegetable supply; angular stomatitis was present, but one would have anticipated a higher percentage incidence. But our observations tend to the conclusion that angular stomatitis is commonly associated with the consumption of a diet largely composed of rice, and deficient in protective foods.

If G 3 and B 2 were to be visited at the time of writing, no stomatitis would be found. In G 3 the condition has disappeared as the result of the addition of 1.5 oz. of skim-milk powder to the original diet, while in B 2 some cases have been cured by unheated and autoclaved yeast and the remainder by a daily intake of 1.0 oz. of skim-milk powder (Aykroyd and Krishnan, 1936c).

Present knowledge of the distribution in foodstuffs of what may be called the 'anti-stomatitis' vitamin can be summarized as follows:—

Milled rice	..	..	..	Absent.
Millet	..	..	..	Present.
Milk, including skim-milk	..	..	..	Abundantly present.
Yeast (unheated)	..	..	..	" "
Yeast (autoclaved)	..	..	..	" "
Liver	..	..	..	" "

#### PHRYNODERMA.

The literature relating to this condition has been summarized by Radhakrishna Rao (1937). A number of workers support the view that it is due to vitamin-A deficiency. In the present investigation we have been unable to detect any specific relation between the frequency of phrynoderma and the composition of the diet, beyond the fact that in general it was most common in the hostels in which the diet was most deficient in protective foods. The figures of carotene and vitamin-A intake are based on the spectrographic assay of particular samples of each foodstuff. The carotene intake estimates are very rough, since the richest source of carotene was green leafy vegetables, which is just the food the intake of which was most difficult to estimate accurately and varies most from season to season. Further the carotene content of any vegetable foodstuff may fluctuate within wide limits, depending on the period of storage, etc. (De, 1936).

Our observations are not incompatible with the theory that vitamin-A deficiency is one of the causative factors of phrynoderma, but the irregularity of its appearance in groups of malnourished children suggests that some other factors may play a part. In B 8, B 9, and B 10, intake of vitamin A and carotene was low, while phrynoderma was absent in B 8, and present in only a small percentage of boys in B 9 and B 10.

Angular stomatitis and phrynoderma are not caused by the same dietary deficiencies. The incidence of the former, due to deficiency of some factor or factors in the vitamin-B<sub>2</sub> complex, showed no correspondence with that of the latter.

## BITOT'S SPOTS.

Bitot's spots is the name usually given to the patches of foamy yellowish-white exudate, appearing on an area of conjunctival xerosis. It was stated at the beginning that we hoped to be able to correlate the frequency of Bitot's spots and the vitamin-A activity of the diet, and hence to throw light on the problem of minimum human requirements of vitamin A and carotene. This expectation has not been fulfilled. A few cases of Bitot's spots were found in nearly all the hostels, but there was no relation between vitamin-A activity as estimated on the basis of spectrographic assays, and the incidence of this eye lesion. As the result of a number of scattered observations we incline to the view that Bitot's spots are a chronic condition which does not readily disappear unless some rich source of vitamin A (e.g., cod-liver oil) is given regularly for some time. Possibly the children with Bitot's spots at the period of inspection showed the same lesion on admission to the institution, resulting from deficient feeding in the earlier years of childhood, and the average hostel diet was not sufficiently rich in vitamin A to cause its disappearance. It is noteworthy that B 4, in which the highest incidence of Bitot's spots was found, is largely a home for destitute orphans. Lieut.-Colonel R. E. Wright, C.I.E., I.M.S., of the Government Ophthalmic Hospital, Madras, has seen occasional cases of Bitot's spots in adults which persist in spite of the consumption of a good diet for many years. More satisfactory evidence about minimum vitamin-A and carotene requirements would be obtained if an *outbreak* of night-blindness and xerophthalmia in an institution could be observed and related to the vitamin-A activity of the diet. The detection of very early commencing signs of xerophthalmia and night-blindness would be of more value, for the establishment of minimum vitamin-A requirements, than the detection of the more chronic condition. The former would require considerable ophthalmological experience in differential diagnosis; Bitot's spots can easily be observed by non-ophthalmologists and we thought it wiser to confine our investigations to a sign in the recording of which there were fewer possibilities of error. We did not study the incidence of night-blindness because of the difficulty of obtaining a satisfactory account from children about a subjective symptom.

In the majority of hostels in which Bitot's spots were observed, vitamin-A and carotene intake was below 2,000 International Units. One  $\gamma$  of carotene and 1 $\gamma$  of vitamin A, as estimated by De's (1937) method, are roughly equivalent to 1.0 and 2.6 International Units respectively. In the hostel in which the vitamin-A activity of the diet was estimated at the lowest figure (B 8), no cases of Bitot's spots were observed; this was a small hostel in which the boys were of a higher average age than the general average of the hostels. In G 1, G 12, and G 13 a few cases of xerophthalmia were present in spite of an estimated intake exceeding 2,500 International Units. In the two latter hostels the relatively high intake was due to the inclusion of sheep's liver in the diet, one or two meals per week being given in lieu of meat. Two samples of *uncooked* liver investigated in these Laboratories have been found to contain about 100 $\gamma$  of vitamin A per gramme. It is to be observed that even 6,500 International Units, roughly the estimated intake in G 12, are less than the quantity of vitamin A which would be provided by 0.5 oz. (15 g.) of average cod-liver oil taken daily. We have observed the improvement of a case of chronic xerophthalmia with Bitot's spots which was given roughly this amount of cod-liver oil for 2 to 3 months, as an addition to a

diet of unknown vitamin-A activity. There is an obvious difference between consuming vitamin A provided in the form of steady daily dose of cod-liver oil and provided in the form of one or two meals of cooked liver per week.

We make no attempt to draw from these investigations deductions about minimum or optimum vitamin-A requirements which could be applied in establishing a standard for purposes of practical dietetics. We simply record the persistence of cases of Bitot's spots on diets which appear, in general, to be of low vitamin-A and carotene content, and particularly deficient in vitamin A. The possible errors of the estimates given must not be minimized.

#### DISCUSSION.

A few remarks on certain practical aspects of these investigations will not be out of place. In return for co-operation on the part of the superintendents of the various hostels, we gave advice about the improvement of diet schedules. Here we were faced with the main problem of practical nutrition work in India; how to make 1 anna do the work of 3 annas. Most of the hostels could afford to increase expenditure on food very little or not at all; it was, therefore, in most cases useless to recommend any of the 'cheap balanced diets' previously described (Aykroyd and Krishnan, 1936*a*) which cost about Rs. 5 per month for an adult and very little less for growing children. To draw up a diet schedule costing Rs. 3-8 per child per month which is thoroughly satisfactory in the light of modern knowledge is impossible. It would have been quite useless in most cases, for example, to offer the recommendation that 8·0 oz. of whole milk should be added to the diet.

There are, however, certain improvements which can be made without great increase in cost. Many of the diets were low in fat; the addition of extra vegetable oil or coco-nut at the expense of a quantity of cereal supplying an equivalent number of calories, does not greatly put up expenditure. (Ghee or butter would be out of the question.)

If the cereal consumed is milled rice, an improvement in the nutritive value of the diet can be brought about by wholly or partially substituting whole rice, whole wheat, or one of the millets; this change involves little addition in cost. It is unfortunate that there is a social prejudice against ragi in South India; it is often regarded as the food of the poor villager—also as the food of prisoners in gaols—and adolescent country boys and girls, who are hoping to raise themselves above the status of their parents, feel entitled to consume the cereal of the townsman and the educated classes—milled rice. Many of the hostel superintendents told us that they had the greatest difficulty in persuading the children to eat ragi and other kinds of millet, even when millet is the staple food of the district from which they come. A further difficulty lies in the fact that ragi is often prepared in an unpalatable form. We have nevertheless succeeded, in some cases, in increasing considerably the millet content of the diet at the expense of rice, with definite improvement in the well-being of the children.

The pulses are relatively inexpensive—in many instances we recommended an increase in intake of 1 oz. to 2 oz. per child per day. Soya bean in small quantities has been introduced into the schedules of one or two hostels.

The consumption of green leafy vegetables can be encouraged. The cheaper varieties—amaranth leaves, coriander leaves, drumstick leaves, etc.—are as nutritious as the more expensive ones, such as lettuce. Here and there, where circumstances were favourable, we suggested the creation of a vegetable garden, to be tended by the children themselves. If advantage is taken of seasonal cheapness, some fruit can usually be supplied with fair regularity at no great expense.

Even a little milk is better than none. An adequate supply of whole milk may be impossible, but a little butter-milk or skim-milk will enhance the nutritive value of hostel diets. In certain institutions we have introduced, with enormous benefit to the children, fairly large quantities of skim-milk reconstituted from New Zealand skim-milk powder, which costs roughly one-third the price of a similar quantity of fresh whole milk. Special mention may be made of one large children's institution, not included in this investigation, in which intake of milk was increased by the *creation* of a cheap and regular local supply, i.e., by ensuring local producers of a regular market. This institution was, however, in a rather better financial position than the majority, and situated in a district favourable for milk production.

There is a large field for useful work in improving diets in children's residential institutions in India. While the problem of malnutrition among day-school children is very difficult to attack, there is no reason why children in hostels should not receive a moderately satisfactory diet.

#### SUMMARY.

(1) The composition of the diets of 24 residential children's hostels in South India has been studied. The diets supplied in such institutions, while in general of low nutritive value, are superior to those consumed by the general population.

(2) Children in hostels are superior in weight for a given height to children of the same class in the general population.

(3) Angular stomatitis, phrynodema, and xerophthalmia were found to be common. The highest incidence of angular stomatitis was observed in hostels in which the diet was largely composed of rice and lacked 'protective' foods. Present knowledge about the distribution in foodstuffs of the 'anti-stomatitis' vitamin is summarized.

(4) No specific relation was observed between the frequency of phrynodema and the composition of the diet, beyond the fact that in general it was most common in hostels in which the diet was most deficient in protective foods. It is difficult to explain the incidence of this condition on the hypothesis that it is due *solely* to vitamin-A deficiency.

(5) Cases of Bitot's spots were found in the majority of hostels. In general, the diets were low in vitamin-A activity, but no precise relation appeared to exist between the vitamin-A activity of the diets and the frequency of Bitot's spots. The significance of this condition as evidence of malnutrition is discussed.

(6) Useful practical work can be done in improving diets in children's residential institutions. While it is often impossible, with available financial resources, to introduce a really satisfactory diet schedule, various improvements can be made with little increase in cost.

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## 'PHRYNODERMA'—A CLINICAL AND HISTO-PATHOLOGICAL STUDY.

· BY

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DURING recent years increasing attention has been paid to the study of the cutaneous manifestations in food deficiency disease. A peculiar dry, scaly consistency of the skin has been described in association with the ocular symptoms by most of the writers on keratomalacia. A detailed clinical description of the changes in the skin in cases of keratomalacia in adults was given by Pillar (1929a and b), who emphasized that keratomalacia is a 'system disease' of the ectodermal tissues, manifesting symptoms not only in the eye but in many parts of the body. Infections of the skin, such as pustular eruptions, furunculosis, abscesses, impetigo, acute bed-sores (in babies), etc., have often been noted in association with keratomalacia. In summarizing the available literature on the subject, Mackay (1934) observed that 'there is no one type of skin infection particularly characteristic of vitamin-A deficiency, but that a variety of skin infections, often chronic or relapsing in character, may occur'.

Besides the above-mentioned changes, a follicular keratosis of the skin has been described occurring in association with xerophthalmia and keratomalacia. Frazier and Hu (1930, 1931) reported the occurrence of spinous papules at the sides of the hair follicles, mostly on the extensor surfaces of the extremities, shoulders, and lower part of the abdomen, in association with a dry and sometimes dark skin, in a group of Chinese soldiers (in a Military Camp near Peiping), who showed typical signs of keratomalacia but no signs of other vitamin deficiency disease. Histologically, the pathological process was one of 'hyperplasia and hyper-keratinization of the epithelium of the epidermis and hair follicles, with associated metaplasia of the glandular structures of the skin and infection'. As a result of providing a well-balanced diet and administering 30 c.c. of cod-liver oil daily, and without any local medication, the papular lesions slowly disappeared leaving delicate atrophic pigmented scars. On the analogy of the pathological changes occurring in the other organs in human and experimental avitaminosis-A, and from the results of the therapeutic tests, these authors concluded that the condition was attributable to a deficiency of vitamin A in the diet. Further clinical and pathological observations on the part of these workers (Frazier and Hu, 1934, 1936) tend to confirm

their original observations on the specific nature of the follicular hyper-keratosis. They have further pointed out that the follicular lesions were most common in young adults and may occur in the absence of the classic ocular signs of vitamin-A deficiency.

A dermatosis, similar to that mentioned above, was described by Loewenthal (1933*a* and *b*) in East Africa. During an inspection of the Uganda Central Prisons, it was found that the majority of a group of prisoners suffering from night-blindness and xerophthalmia also had the cutaneous eruption. Treatment with one ounce of cod-liver oil a day, and without any modification of the diet, for a period of nine weeks, resulted in the cure of night-blindness and xerophthalmia in every case and of the dermatosis in about 98 per cent of cases. The condition was regarded as a dyskeratosis, with changes consequent on this condition; no signs of sepsis or softening were noticed. It was also pointed out that the histological changes were precisely the same as those occurring in the papules of *Pityriasis rubra pilaris*, although the anatomical distribution of the two conditions was different. The dermatosis was attributed to a deficiency of vitamin A in the diet. The papular eruption was later taken as one of the criteria of vitamin-A deficiency in the inspection of schools (Loewenthal, 1935*a*) and was subsequently included under the accepted signs and symptoms of vitamin-A deficiency (Loewenthal, 1935*b*).

Similar, but independent, observations were made by Nicholls (1933) on the occurrence of a papular dry skin, which was frequently accompanied by mild neuritis, keratomalacia or diarrhoea, among poorly fed labourers in East Africa and also among convicts in a jail in Ceylon. He gave the condition the name of '*phrynoderma*' (toad-skin) (Nicholls, 1933, 1934, 1935 and 1936). He attributed it primarily to a vitamin-A deficiency in the diet, but considered that deficiency of other food factors may also be responsible. He further pointed out that phrynoderma, which is a sign of dietary deficiency, is of great value in determining the 'state of nutrition' of groups of persons (Nicholls, 1935).

Goodwin (1934) in London, and Sweet and K'ang (1935) in Peiping (China), described similar follicular keratotic lesions in association with vitamin-A deficiency. Wright (1936) in Madras (India) described papular skin lesions similar to those of *Pityriasis rubra pilaris*, occurring on elbows and knees, both in adults and children suffering from keratomalacia. Giblin (1936) has long observed cutaneous manifestations, similar to those described by Loewenthal (1933*b*), associated in some cases with xerophthalmia, in the syndrome of vitamin-A deficiency in Papuan natives. Wright, E. J. (1930), describing 'A and B avitaminosis disease' in Sierra Leone, mentioned a keratosis of the skin of the limbs and trunk, the skin becoming dry and rough.

The incidence of phrynoderma in groups of school children in South India has recently been reported from these Laboratories by Aykroyd and Rajagopal (1936). In this paper, the clinical manifestations and the histo-pathological features of the condition have been described and its ætiology is discussed.

#### CLINICAL OBSERVATIONS.

The clinical observations described in this paper were made on children attending the local schools and the Municipal Child-Welfare Centre, and on adults and children in the Government Lawley Hospital, Coonoor. Their ages varied

from 3 to 35 years. Most of them belonged to the poorer classes and the diet which they obtain in their homes usually consists mainly of milled rice, a small quantity of various kinds of pulses and vegetables with condiments and a little millet; 'protective' foods such as milk, milk products, meat, eggs, etc., are consumed only in very small quantities occasionally or not consumed at all.

The general condition of the individuals showing phrynoderma, taking the group as a whole, was poor. A 'chubby' child was, however, occasionally seen with no other signs of ill health apart from the cutaneous manifestations. In some instances, especially in children, phrynoderma was associated with 'angular stomatitis'. The association of typical 'Bitot's spots' is not common, but varying degrees of irregular xerosis, loss of lustre, discoloration, or in some instances wrinkling, of the conjunctiva, were often present. The skin and, in most cases, the hair were generally dry.

### *The papular eruption.*

(a) *Onset*.—In most instances the onset was insidious, some of the patients being unaware of its existence till the time of examination. A dry and slightly rough skin was sometimes noticed preceding the appearance of the typical papules.

(b) *Subjective symptoms*.—Subjective symptoms were absent and, even in response to leading questions, no pain or itching was complained of, and there was no tenderness.

(c) *Site*.—The papules were situated at the site of the pilo-sebaceous follicles, and microscopic examination revealed that the lesions were mainly in and around the latter.

(d) *Distribution*.—In most instances the papules were roughly symmetrical in their distribution and were either profuse or localized. They were generally discrete and tend to appear in groups. The papules were present mostly on the extensor surfaces of the arms and thighs (see Plate XXVI) and the upper part (postero-lateral aspect) of the forearms near the elbows; in some they were also present on the extensor surface of the upper part of the legs, posterior axillary folds, over and in between the scapular regions, and across the buttocks and flanks. Acne-like eruptions on the face were not a conspicuous feature, although the skin in this situation was also dry as in the other areas. The genito-anal regions and the hands and feet were not affected by the papular eruption.

(e) *Description of the papules*.—The papules varied in size from a big pin's head to a millet seed, and were rounded or hemispherical in outline with sharply defined edges (see Plate XXVI). Their surface was slightly rough and presented a central keratotic plug, which either projected from the atrophied hair follicle or was surmounted by a loosely adherent scale. The colour of the papules resembled that of the skin but, in some instances, hyper-pigmentation was evident in, and immediately around, the papules. The papules were firm in consistency; in some children in whom the papular eruption resembled an exaggerated and permanent 'goose skin' (*Cutis anserina*), the papules were not hard as in the others.

(f) *Clinical examination*.—Phrynoderma was present in children of both sexes as well as in adults. To the touch the affected areas gave a sensation of passing over a nutmeg grater surface. Sensation in response to light touch, pin-prick,

and heat and cold was apparently normal in the areas of skin affected by the eruption.

(g) *Course and evolution*.—The papules showed no tendency to pustulation. In one case—a boy aged 2½ years with spinous papules in most of the situations described above—which improved under treatment with cod-liver oil (half an ounce twice daily) and a well-balanced diet for a period of about two months, small delicate cicatrices were noticed in areas from which the papules had disappeared. It is hoped to undertake further therapeutic tests on cases of phrynoderma and observe its course under treatment.

#### HISTO-PATHOLOGICAL FEATURES.

Material for histo-pathological study has been obtained by biopsy from ten hospital patients, showing typical papules. Serial sections of the skin were cut in each instance and stained by Ehrlich's acid-hæmatoxylin and eosin, Weigert's iron-hæmatoxylin and van Gieson's stain and orcein.

(i) *Epidermis*.—There was a superficial hyper-keratosis of the epidermis in all cases; in places, the *stratum corneum* was very much broadened (see Plate XXVII, figs. 3 and 4). The keratin was either homogeneous in structure or present in loose meshes. There was no evidence of para-keratosis.

No appreciable changes were present in the *stratum lucidum* and the *stratum granulosum*.

The *rete mucosum* in some places, especially in the neighbourhood of the atrophied hair follicles, showed moderate hypertrophy (see Plate XXVII, figs. 5 and 6). The interpapillary processes were widened and more prominent in these areas. The prickle cells appeared normal but, in places, showed vacuolation in their protoplasm.

The cells of the *stratum germinativum* were apparently normal.

Evidence of hyper-pigmentation was not marked even in the neighbourhood of the papules; in some places, however, the pigment-bearing cells were increased in number and were distributed throughout the thickness of the *rete mucosum*.

(ii) *Corium (cutis vera)*.—But for some of the changes described below in the perifollicular regions, the corium (*cutis vera*) was apparently normal. In three cases, however, there was slight perivascular round-celled infiltration. Otherwise there was no congestion, no cedema or hæmorrhages. The fibrous and elastic tissues (see Plate XXIX, fig. 11) appeared normal. Very few chromatophores were present in the superficial layers of the corium.

(iii) *Sebaceous glands*.—The sebaceous glands were not seen in connection with the atrophied hair follicles (see Plate XXVIII, fig. 10; Plate XXX, figs. 15, 16, and 18). Serial sections, however, revealed that the glands were still present in some areas in which the follicular lesions were not very marked; in these areas there was a varying degree of hyper-keratinization of the lining epithelium of the funnels of the hair follicles, and the sebaceous glands showed either a decrease in the amount of cytoplasm or in the number of cells (see Plate XXVIII, figs. 7 to 10). At a later stage of the follicular lesion, a few cells with very little cytoplasm represented the glands; in the typical papules they were atrophied and their site was generally occupied by young connective tissue and mononuclear lymphoid cells.

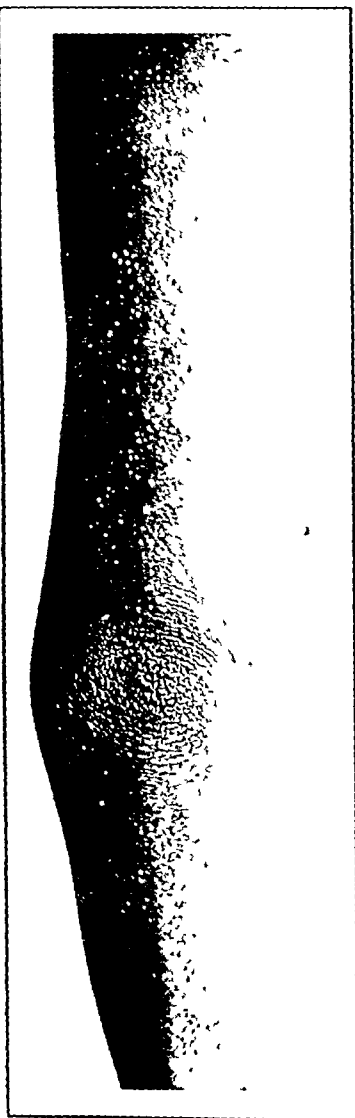


Fig. 1.



Fig. 2.

Figs. 1 and 2. Photographs showing the papular cutaneous lesions on the extensor aspects of the limbs in a boy aged 11 years. Note the ichthyotic dry skin on the leg.

[All the photomicrographs were taken with 'Miflex' (Zeiss)].



Fig. 3.



Fig. 4.

and 4. Superficial hyper-keratosis of the epidermis; note the broadening of the *stratum corneum*. An atrophic hyper-keratotic hair follicle is seen on the right in Fig. 4.  $\times 50$ .



Fig. 5.

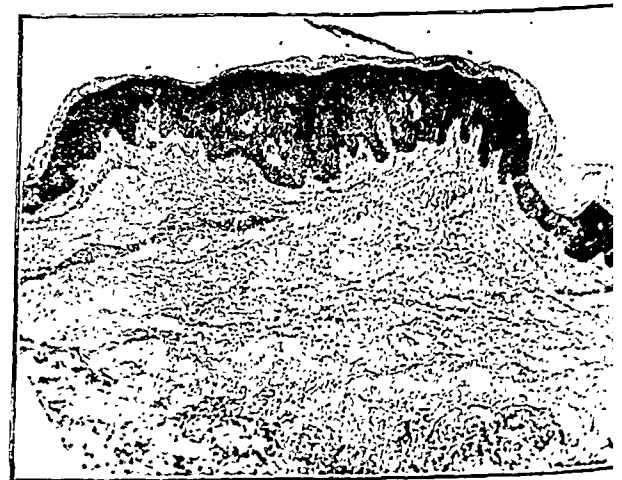


Fig. 6.

Figs. 5 and 6. Epidermis showing epithelial hyperplasia.  $\times 50$ .

PLATE XXVIII.



Fig. 7.



Fig. 8.



Fig. 9.



Fig. 10.

Figs 7 to 10. The lining epithelium of the funnels of the hair follicles shows varying degrees of hyper-keratinization; the sebaceous glands, which are still present, show a decrease in the amount of cytoplasm or in the number of cells (the sebaceous gland in Fig. 7 is almost normal). An advanced stage of follicular lesion is seen on the left in Fig. 10. (Fig. 10. Weigert's iron-haematoxylin and van Gieson's stain).  $\times 50$ .



Fig. 12.

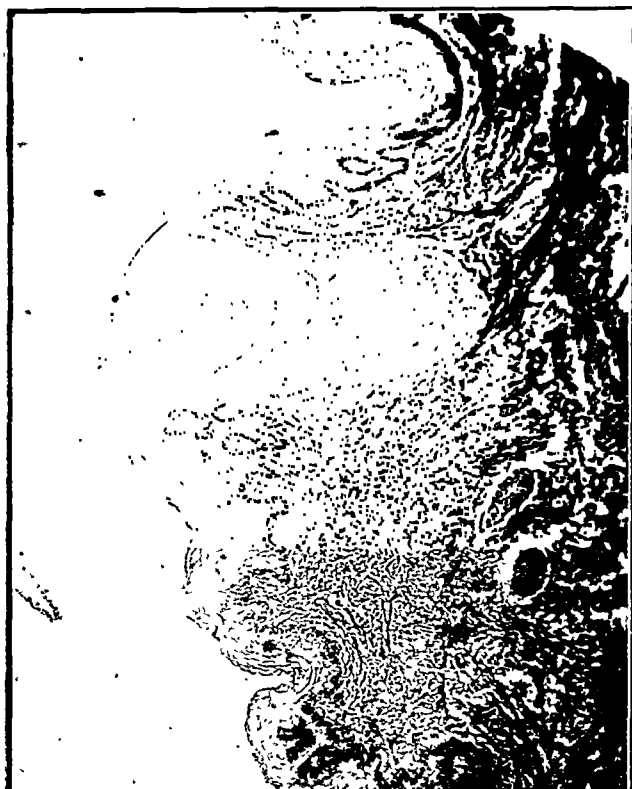
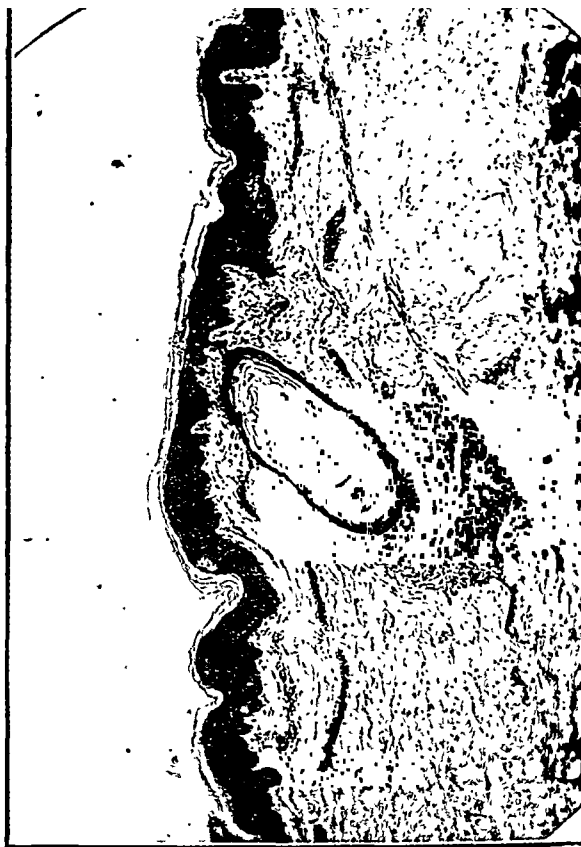


Fig. 11.





### EXPLANATION OF PLATE XXIX.

- Fig. 11. Section of a papule showing the normal distribution of elastic tissue in the corium. The elastic fibres are stained dark; Orcein.  $\times 50$ .
- „ 12. Group of sweat glands showing almost normal appearances. The changes in the hair follicles in this case are shown in Plate XXVIII, fig. 8, and Plate XXX, fig. 17.  $\times 100$ .
- „ 13. Section of skin showing the filter-like depression at the mouth of the hair follicle, and the sebaceous glands. Note the absence of hyper-keratosis of the lining epithelium of the funnel of the follicle. The skin clipping was taken from a labourer with an apparently normal skin.  $\times 50$ .
- „ 14. Section of a hair follicle from a case of phrynoderma showing the mononuclear lymphoid cells in the perifollicular region, mostly in the lower part.  $\times 50$ .

#### EXPLANATION OF PLATE XXX.

Figs. 15 to 18. Sections of papules showing hyper-keratosis of the hair follicle dilatation of their funnels by the keratotic plugs and cellular infiltration in the perifollicular tissues. Remnants of the sebaceous glands can be seen in Fig. 17. Note the coiled and atrophic hair, cross sections of which can be seen in the substance of the follicular plug in Fig. 16, and the atrophic lower part of the follicle, separated from the upper part by delicate fibrous tissue, in Fig. 18.  $\times 50$ .



Fig. 16.



Fig. 16.



Fig. 16.



Fig. 17.



(iv) *Sweat glands*.—Changes in the sweat glands were not a marked feature (see Plate XXIX, fig. 12). In some instances, however, the coil glands were few in number, their epithelium was flattened or shrunken and irregular, and the lumen dilated; the cells were not granular in appearance and the general impression obtained after studying the serial sections was that the glands were not actively secreting. Slight round cell infiltration around the sweat glands was present in one instance. The funnel-like depressions in the epidermis containing the sweat pores was filled to a varying extent with the hyper-keratinous material. The spiral cleft between the prickle and horn cells, representing the upper part of the sweat duct, was sometimes found occluded by cornified material or was surrounded by cells containing keratohyaline granules. In one case, the upper part of some of the ducts in the corium was surrounded to a varying extent by bands of epithelium from the epidermis.

(v) *Hair follicles and hair*.—Marked changes were found in the hair follicles in all instances; the number of follicles affected and the degree of changes present, however, varied. The mouths of the hair follicles showed marked hyper-keratinization of the lining epithelium; as a result, the funnels of the follicles were widened and were plugged by dense masses of horny tissue (see Plate XXX, figs. 15 to 18). Closer examination revealed that the follicular plugs consisted of concentric lamellæ of flattened cornified cells in which there were no nuclei; in one case, however, the outer layers of the plug, which generally were not dense, showed few persistent nuclei. Instead of the filter-like depression (see Plate XXIX, fig. 13), slight or marked elevations were often found at the mouths of the affected follicles. Sections of the coiled and atrophic hairs (see Plate XXX, fig. 16) or broken hairs were seen in the substance of some of the follicular plugs, but pustulation in the latter was not noted in any instance.

Serial sections showed that the lower part of the affected follicles was atrophic and was often separated from the upper part of the follicles by delicate fibrous tissue (see Plate XXX, fig. 18). In some places, however, the lower third of the follicles showed moderate hypertrophy of the external root sheath. Pieces of atrophied hairs were seen in the lower part of some of the follicles.

Varying degrees of cellular infiltration—mostly fibroblasts and mononuclear lymphoid cells—were often present in the loose perifollicular tissue; poly-morpho-nuclear leucocytes, eosinophils, and evidences of hæmorrhage were not noticed in any instance (see Plate XXIX, fig. 14, and Plate XXX, figs. 15 to 18).

Briefly described, the main histo-pathological features of this condition consisted of a superficial hyper-keratosis of the epidermis and hair follicles, marked dilatation of the funnels of the pilo-sebaceous follicles by keratotic plugs, atrophy of the sebaceous glands, and impaired function of the sweat glands.

#### COMMENT.

It may be seen from the above description that the clinical and the anatomical features of phrynoderma, as met with here, are similar to those described by Frazier and Hu, Loewenthal, Nicholls, and others. The follicular lesions were reported to be frequent in young adults by Frazier and Hu (1934, 1936); in the present series of cases, however, the condition appeared to be equally common in

children of both sexes, the youngest being a child of 2½ years. Loewenthal (1935a), Nicholls (1934) and Wright (*loc. cit.*) found this condition common in children.

The papules, which constitute the important clinical manifestation of the condition, arise from pilo-sebaceous follicles as a result of a non-inflammatory hyper-keratosis of the lining epithelium. The mouths of the follicles gradually become dilated and plugged by the dense masses of horny tissue and the resulting mechanical pressure appears to be responsible for the atrophy of the lower part of the follicles. The perifollicular infiltration is probably secondary to the irritation of the keratotic plug in the orifice of the hair follicle. From the histological examination, atrophy of the sebaceous glands appears secondary, the primary pathological feature being hyper-keratinization of the epidermis and hair follicles. As pointed out before, sebaceous glands could still be seen in some follicles, the mouths of which showed varying degrees of hyper-keratinization (see Plate XXVIII, figs. 7 to 10). The absence of sebaceous cysts in connection with the damaged follicles in all instances would suggest that, apart from the mechanical factor, the nutritional deficiency is also primarily responsible for the atrophy of the sebaceous glands. Bommer (1934) attributed atrophy of sebaceous glands in the skin to a deficiency of vitamin A in the diet and Nicholls (1934) thought that the sebaceous glands require a fat-soluble vitamin for their secretion. The absence of marked pathological changes in the sweat glands suggests that the absence of sweating noted in the areas of the follicular lesions is mostly due to functional disturbances in the coil glands.

Similar follicular keratotic lesions have been described by Nicolau (1918), Wiltshire (1919), Aschoff and Koch (1919), Theodorescou (1928), Scheer and Keil (1934), and other workers in cases of scurvy. In reviewing the literature on the subject, Scheer and Keil (*loc. cit.*) pointed out that in scurvy the vascular lesions, which are the characteristic feature of the disease, are mainly present in the capillaries of the perifollicular region, and the hyper-keratosis which results is mainly limited to the hair follicles; on the other hand, the earlier follicular lesions of scurvy may not be distinguishable clinically from those observed in *phrynoderma*. A number of other conditions, such as *Ichthyosis follicularis*, *Lichen plano-pilaris*, *Pityriasis rubra pilaris*, *Keratosis supra-follicularis*, *Lichen spinulosus*, etc., characterized by the presence of horny plugs at the orifices of the hair follicles forming small papules, have been described (Macleod, 1920). It is, therefore, desirable to establish, beyond doubt, the specific nature of *phrynoderma*.

As previously noted, most observers have described *phrynoderma* as occurring in association with xerophthalmia and keratomalacia. The beneficial results of the therapeutic administration of cod-liver oil on the papular eruption, reported by Loewenthal (1933a and b) and Frazier and Hu (1931, 1934, and 1936), strongly suggest that the cutaneous manifestations are part of the syndrome of avitaminosis-A, and are not merely coincident symptoms. The primary non-inflammatory hyper-keratosis of the epithelium of the epidermis and hair follicles in *phrynoderma*, and the involvement of the cutaneous glands and hair derived from the ectoderm, are similar to the morphologic appearances seen in vitamin-A deficiency. On the other hand, investigations carried out by workers in these Laboratories (Aykroyd and Rajagopal, *loc. cit.*; Aykroyd and Krishnan, 1937) show that it is difficult to explain the incidence of *phrynoderma* on the hypothesis that vitamin-A deficiency is the sole cause. Thus, the condition is very rare in school children in Calicut on

the west coast of Madras, although xerophthalmia is rather more common than in other districts. Investigations in children's hostels revealed no definite association between the frequency of phrynoderma and xerophthalmia, and between the former and vitamin-A and carotene intake. Loewenthal (1933b) pointed out that in persons on an almost fat-free diet which is rich in vitamin-A content, the papulo-follicular dermatosis was not seen.

Experimental evidence which would throw light on the relation between cutaneous lesions as described and diet deficiency is lacking. Smith (1932) and Smith and Sprunt (1935) noted atrophy of the sebaceous glands in the tails of vitamin G (B<sub>2</sub>)-deficient rats. Similar but less marked changes were also noted in vitamin-A and vitamin-B<sub>1</sub> deficiency (Smith and Sprunt, *loc. cit.*). Further experimental work would probably throw some light on the subject.

The absence of vascular changes or hæmorrhages in the perifollicular tissues, even in the advanced stages of the papular eruption in phrynoderma, rules out the anti-scorbutic factor from the ætiology of the condition. The clinical features of *Ichthyosis follicularis*, *Lichen plano-pilaris*, *Pityriasis rubra pilaris*, and *Acne vulgaris* are different from those of phrynoderma, so that difficulty rarely arises in the differential diagnosis of the latter. In the light of present knowledge, it would be interesting to study the ætiology of some of the conditions, such as *Lichen pilaris* (Smith, E. C., 1932), *Keratosis supra-follicularis*, *Lichen spinulosus*, etc., described as separate entities.

It is generally agreed that phrynoderma is a separate entity associated with malnutritional states. But, in view of divergent opinions regarding its ætiology, further study is required to determine the exact nature of the condition.

#### SUMMARY AND CONCLUSION.

The clinical and histo-pathological features of a papulo-follicular dermatosis described as '*phrynoderma*', associated with malnutritional states, were studied. The condition was found both in children and adults, mostly on the extensor surfaces of the arms, thighs and upper part (postero-lateral aspect) of the forearms near the elbows. An acne-like eruption on the face was not a conspicuous feature.

Histologically, the condition was characterized by a superficial non-inflammatory hyper-keratosis of the epithelium of the epidermis and hair follicles, distension of the mouths of the pilo-sebaceous follicles by horny plugs, atrophy of the sebaceous glands and impaired function of the sweat glands.

The condition is probably a manifestation of a nutritional deficiency in which lack of vitamin A is an important factor.

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## THE SPECTROPHOTOMETRIC METHOD OF ASSAYING VITAMIN A AND CAROTENE WITH FURTHER DATA ON THE VITAMIN-A ACTIVITY OF INDIAN FOODSTUFFS.

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In a previous communication (De, 1935), a simple spectrophotometric method of assaying vitamin A and its precursor carotene was described. Subsequently the method was, with slight modifications, applied in the assay of the carotene content of over 100 samples of vegetable foodstuffs (De, 1936*a* and *b*). In the present paper, further data regarding this method of assay are presented, and the results of the investigation of the vitamin-A potency of a further 70 foodstuffs are included. During the routine examination of foodstuffs, additional evidence has appeared in support of the validity of the technique and a few more points of technical importance have been brought to light.

Of the various methods of assaying vitamin A and carotene, viz., biological, chemical, and spectrographic, the last named has in recent years been accepted as the most satisfactory (Morton, 1935 ; and other workers). In vitamin-A assay work by non-biological laboratory methods, it is necessary that vitamin A and carotene should be estimated separately. There is no satisfactory chemical method for distinguishing and estimating them separately when they occur simultaneously. Double estimations are, however, needed only in the analysis of animal products in which vitamin A and carotene occur together. In the case of vegetables, vitamin A as such does not occur (Wolff *et al.*, 1930 ; Moore, 1931 ; De, 1935 ; and other workers) ; hence carotene alone has to be estimated.

Although vitamin A and carotene resemble each other in their physiological action, they are quite different in their chemical and physical properties. As regards molecular constitution, vitamin A is an unsaturated alcohol (colourless), while carotene is a yellow unsaturated hydrocarbon pigment. The former shows a characteristic absorption spectrum with a single maximum at 3,280 A. U. The latter (like the xanthophylls and many other lipochrome pigments) besides its specific absorption in the visible region shows some weak bands in the long and

short ultra-violet. Estimation of carotene from studies of absorption in the visible region is not influenced by the presence of vitamin A, while an accurate assay of vitamin A contained in animal products, particularly richly pigmented ones (butter, egg-yolk, blood, etc.), is rather a complicated problem, as the carotenoids occurring simultaneously may markedly influence the gross absorption at 3,280 A. U. To avoid this difficulty two courses can be followed: (1) to deduct from the gross absorption at 3,280 A. U. that contributed by the carotenoid pigments, or (2) to devise a simple chemical process that will isolate vitamin A from the interfering pigments.

#### DEDUCTION OF ABSORPTION DUE TO THE CAROTENOIDS.

Gillam (1934) has used this principle in estimating vitamin A contained in various samples of butter. The gross intensity of absorption at 3,280 A. U. was measured; this was in part due to vitamin A and in part due to carotene accompanied by traces of xanthophylls. The absorption at 3,280 A. U. due to the latter was obtained by dividing the  $E \frac{1 \text{ per cent}}{1 \text{ cm.}}$  value at 4,500 A. U. to 4,600 A. U. by 6.5. In the present experiments absorption spectra of several solutions of pure carotene (International Standard Preparation, 1931) were studied and a similar ratio (1/6 to 1/7) between the intensities of absorption at the region of 3,300 A. U. and 4,500 A. U. to 4,600 A. U. was recorded. This procedure of estimating the non-vitamin absorption will serve well, provided the interfering pigments consist mainly of carotene as in the case of butter. In the analysis of other test: ... (etc.) where carotene is associated with high proportions of xanthophylls and similar pigments, it would hardly be justifiable to evaluate the irrelevant absorption in the same way. We have little knowledge regarding the true value of the correction factor that would be applicable in such complicated cases; and any attempt to remove these non-hydrocarbon pigments by the Kraus separation will simultaneously remove part of the vitamin A present.

#### PARTITION BETWEEN VITAMIN A AND CAROTENE BY THE PHASE TEST.

It has been reported by several workers (Wolff *et al.*, *loc. cit.*; Rosenthal *et al.*, 1935; and other workers) that vitamin A and carotene can be separated from a petroleum ether solution by repeated phase tests between alcohol and petrol, vitamin A going to the former solvent while carotene is left in the latter. Experiments were undertaken to study the solubility of vitamin A in alcohol (90 per cent) and petroleum ether (B. P. 60°C. to 80°C.). It was found that vitamin A is highly soluble, and also more or less equally soluble in both these solvents. As such, strictly speaking, the above procedure of depriving petroleum ether of vitamin A by alcohol is not a real Kraus separation. Vitamin A distributes itself more or less uniformly in both the phases and in each operation, with an equal volume of 90 per cent alcohol, roughly half the amount of vitamin A would be removed. It can thus be anticipated that for a quantitative separation (within 1 per cent error), using each time an equal volume of alcohol, at least seven operations would be needed, and the percentage concentration of vitamin A in petrol would fall roughly in the following geometrical progression: 100, 50, 25, 12.5, 6.25, 3.12, 1.56, 0.78, and so on. This

has been experimentally confirmed as follows : petroleum ether solutions of vitamin A (unsaponifiable fractions of halibut-liver oil) were washed repeatedly with an equal volume of 90 per cent alcohol (ethyl and methyl) until no vitamin A was left behind in the petrol as tested colorimetrically. For a quantitative partition as many as 7 to 10 washings were required. Partition in the reverse direction—alcohol to petrol—was also investigated and found to be equally effective. Recently, Gillam and Senior (1936) have investigated this problem thoroughly, and report similar results which were published while the manuscript of this paper was being prepared. The partition method was then tried on several petroleum ether solutions of Haliverol dissolved directly. Even after 10 washings with 90 per cent alcohol, only a part of vitamin A was removed from the petrol. This discrepancy is probably due to the fact that petrol, as compared with alcohol, is a much better solvent of fats and as such exerts a stronger pull on the free fat, while the fat-soluble vitamin A preferentially adheres to the parent substance. Similarly, Gillam and Senior (*loc. cit.*) report that the presence of large quantities of sterols (cholesterol) affects the partition co-efficient of vitamin A, petrol retaining the greater portion of vitamin A. The solubility of a commercial sample of cholesterol was investigated and found to be greater in petrol than in alcohol. It can be concluded from these observations that the presence of any foreign material having a greater solubility in petrol will affect the partition in the same way.

The question now arises, how far this partition method may be useful in routine vitamin-A and carotene assay work. The possibility of its use is certainly very much limited, like that of the previous method, since xanthophylls and other pigments will invariably accompany vitamin A to the alcohol phase.

#### REMOVAL OF THE PIGMENTS BY CHARCOAL ADSORPTION.

It has long been known that charcoal is a very powerful adsorbent for various pigments. Experiments were conducted to discover if charcoal adsorption could effect a quantitative separation of vitamin A from the associated pigments. Applications of this method, though not with strict quantitative purposes, have already been mentioned by several workers (Gillam *et al.*, 1933; and others). It was observed that treatment with charcoal destroys both vitamin A and carotenoids, the latter, however, being much more quickly destroyed than the former. Stability to charcoal could be much increased by addition of hydroquinone. Several ether and petroleum ether solutions of vitamin A (cod-liver oil) and carotene (red-palm oil), the proportionate concentrations of which ranged from 1 : 1 to 1 : 5, were treated with a minimum amount of charcoal, slightly shaken, filtered, and the precipitate washed 3 to 5 times, the whole operation being carried out as quickly as possible. In each of these attempts, carotenoids were removed completely, leaving behind a clear colourless solution of vitamin A. There occurred no appreciable loss of vitamin A, as evidenced by the arsenic-trichloride test even when the test solutions contained as low as 2γ to 5γ of vitamin A per c.c. A petroleum ether solution, containing per c.c. 4γ and 20γ of vitamin A and carotene respectively, was decolorized by charcoal (0.2 g. charcoal per c.c. : shaken for 2 minutes, filtering and washing for 8 minutes) and examined spectrographically. The loss of vitamin A in this case was only 9 per cent. This method of separation of vitamin A from all classes of associated pigments appears to be fairly satisfactory.

and with sufficient precautions it may be reliably applied in routine work. Further data about the charcoal adsorption of vitamin A and carotene will be reported later.

#### STABILITY OF VITAMIN A AND CAROTENE TOWARDS LIGHT.

It is a well-known fact that both vitamin A and carotene are subject to destruction by light. It is thus of great technical importance to adjust the conditions so that during the photometric study concentrations of vitamin A or carotene are least affected by exposure to light. Precise knowledge of how this can be done is still lacking, although spectrophotometric methods are nowadays being extensively used. Accordingly, the following investigation was undertaken: roughly equal quantities (about 15.0 $\gamma$ ) of vitamin A and carotene (in cod-liver oil and red-palm oil respectively) dissolved in equal volumes (10 c.c.) of different solvents, viz., chloroform, ether, petroleum ether, and alcohol, were exposed to the total radiations of a quartz mercury vapour lamp (Heraeus: current 3.5 amperes at 220 D. C.) under identical conditions (distance 20 cm., thickness of the layer of solution about 1.5 cm.) and their stabilities to light compared. The evidences for the destruction of vitamin A and carotene were respectively a negative arsenic-trichloride reaction and the disappearance of all yellow colour. The results of several experiments are set forth in Table I:—

TABLE I.

*Effect of ultra-violet irradiation on vitamin A and carotene.*

Solvent.	APPROXIMATE MINIMUM TIME (IN MINUTES) REQUIRED FOR COMPLETE DESTRUCTION: CONCENTRATION, ROUGHLY 15 $\gamma$ PER C.C.	
	Vitamin A.	Carotene.
Chloroform (Merck)	7 to 9	1.5 to 3
Ether (Kahlbaum) ..	23 to 25	35 to 40
Petroleum ether (B. P. 60°C. to 80°C.).	22 to 26	20 to 25
Alcohol (96 per cent ethyl).	20 to 25	22 to 27

From Table I, it is seen that both vitamin A and carotene are easily destroyed by ultra-violet irradiation in all the solvents, and that the effect is very much more rapid in chloroform. The stabilities of vitamin A and carotene are more or

less alike in alcohol and petrol. In chloroform, the photo-chemical destruction of carotene is much quicker than that of vitamin A, while in ether, carotene is slightly more stable than vitamin A (De, 1935). Inactivation by ultra-violet irradiation of vitamin A and carotene in the presence of hydroquinone was also investigated and found to be considerably retarded. Irradiation of carotene by visible light alone was found to be very much less effective, while in the case of vitamin A there occurred practically no destruction (De, 1935).

Solutions containing varying amounts of vitamin A and of carotene in the absorption cells (1 cm. quartz vessels used with the Hilger's sector photometer) were then exposed to the total radiations of the source (spark between Tungsten steel electrodes, gap 4 mm.) under identical conditions as in a photometric study. In all cases an exposure of 10 minutes was given. In chloroform roughly 40 to 95 per cent of the potencies were destroyed, as judged by colour test, even when the concentrations were as high as  $5\gamma$  to  $10\gamma$  per c.c. Chloroform is thus quite unsuitable as a solvent for spectrographic analysis. Even within a few hours, under ordinary conditions, vitamin A and carotene deteriorate appreciably in chloroform. In other solvents, viz., ether, petroleum ether, and alcohol, the loss was not noticeable using concentrations of  $5\gamma$  to  $7\gamma$  per c.c., while with dilute solutions ( $1\gamma$  to  $2\gamma$  per c.c.) the destruction was definitely marked. Exposures to the source, after filtering off the short ultra-violet radiations by interposing a thick transparent glass plate, were found to be absolutely harmless in the same conditions. While assaying carotene by studies of absorption at 4,500 A. U. to 4,600 A. U., it would be preferable to use only the visible radiations.

In order to safeguard the accuracy of the technique, while using the total radiations, it is necessary that the test solutions should neither be too diluted, nor too concentrated, since in both cases appreciable destruction may occur; in the former even a short exposure is enough to affect the concentration, while in the latter longer exposures will be necessary and this may cause much destruction. It appears, however, that previous treatment with pure hydroquinone may solve this difficulty. The addition of a foreign material must not, however, interfere with the study of absorption. To elucidate this question, petroleum ether solutions of hydroquinone, irradiated and non-irradiated, were spectrographed and found to be transparent in the regions concerned.

It may be mentioned that when the concentrations of the test solutions can be previously guessed from the colour or the nature of the material, it would be safer and economical to give only a few exposures, adjusting the variable sector about the position computed, instead of photographing for all its readings. It appears also that concentrations of the order of  $5\gamma$  to  $7\gamma$  of the potent factor per c.c. (giving an extinction co-efficient equal to about 1) are most suitable for spectrography.

c

#### THE ASSAY OF FOODSTUFFS.

##### *Technique.*

The principle and the technique were in general the same as those described previously (De, 1935, 1936a). Test solutions of vitamin A were obtained in ether

or pétroleum éther, those of carotene in the last-named solvent. The amount of vitamin A or carotene present was estimated by determining the difference between the extinction co-efficients at 3,280 A. U. and 4,550 A. U. to 4,600 A. U., respectively, before and after irradiation. The absorption cell was filled with the test solution treated with a little spectrographically pure hydroquinone, while the compensating cell was filled with the extract from which vitamin A or carotene had been completely removed by irradiation for periods just sufficient, under a mercury vapour lamp. For calculations, the following formulæ were used:  $E \frac{1 \text{ per cent}}{1 \text{ cm.}} = 1,600$  for pure vitamin A and 2,500 for pure carotene (Carr and Jewell, 1933; Gillam, 1935). In the photometric study of carotene solutions, the short ultra-violet radiations of the source were filtered off by interposing a thick transparent glass plate.

### *Extraction.*

The vitamin-A assay was carried out on materials of animal origin only, since vegetable foods have been found to be devoid of vitamin A (Wolff *et al.*, *loc. cit.*; Moore, *loc. cit.*; De, 1935; and other workers). Test solutions from potent oils and fats were prepared directly by dissolving them in a suitable solvent. Other materials were saponified on a water-bath under reflux, with slightly excess of colourless 20 per cent alcoholic potash, and the unsaponifiable fraction extracted with light pétroleum ether. After washing several times with distilled water, the extract, freed from moisture, was concentrated to a suitable volume by low heat in an atmosphere of carbon dioxide gas. The final solution, if appreciably coloured, was divided into two parts. Part 1 was decolorized by shaking with a minimum amount of charcoal and used for vitamin-A assay. Part 2 was freed from the non-hydrocarbon pigments by the usual procedure (De, 1935, 1936a) and used for carotene estimation. Direct extraction of vitamin A and carotene by alcohol and petrol was tried on a few animal products. This procedure was not at all encouraging, since the extracts, after concentration, were insufficiently clear and transparent, owing to the presence of too much of fatty substances. This difficulty, due to excess of fats, was not experienced in the case of vegetable foods (except oils very poor in carotene), and carotene was extracted directly with absolute alcohol and then repeatedly with pétroleum ether. The extracts were collected together in a separating funnel, and a few c.c. of water were added, bringing the alcohol to about 85 per cent. On shaking, carotene went to the petrol phase and xanthophylls to alcohol. The carotene solution was then purified from the irrelevant pigments by repeated phase tests, and by adsorption on calcium carbonate.

Several workers have remarked that saponification is necessary to free carotene from the combined xanthophylls and their esters. No appreciable difference was noted in the study of a few test materials (amaranth, red-palm oil, and egg-yolk), with and without saponification. It is possible that these interfering substances do not occur in any significant amount, or probably they are removed by adsorption on calcium carbonate.

The present vitamin-A assay work forms a part and is a continuation of the food survey work in progress in these Laboratories. In this paper data about the

vitamin-A activities of some 70 additional foodstuffs are reported. The results are set forth in Tables II and III :—

TABLE II.

*The vitamin-A and carotene content of some animal products.*

Material.	Vitamin-A content per gramme ( $\gamma$ ).	Carotene content per gramme ( $\gamma$ ).
Adexoline capsules .. .. .	3,125.00 (per capsule).	..
Cod-liver oil (Norwegian) .. .. .	562.50	..
„ (different sample) .. .. .	400.00	..
„ ( „ „ ) .. .. .	314.00	..
„ ( „ „ ) .. .. .	312.00	..
„ ( „ „ ) .. .. .	187.00	..
„ ( „ „ ) .. .. .	156.00	..
Giant-ray-liver oil ( <i>Decerobatus</i> sp.) .. .. .	Trace only	..
Haliverol (Parke, Davis & Co.) .. .. .	12,702.00	..
Saw-fish-liver oil ( <i>Pristis</i> ) .. .. .	170.00	..
Skate-liver oil .. .. .	437.00	..
Chamban (whole fish) .. .. .	Trace only	..
Dolphin oil (from head and brain) .. .. .	Trace only	..
Herring oil ( <i>Clupea toli</i> ) (whole fish) .. .. .	1.00	..
Mackerel oil ( <i>Rastrilliger-kanangurta</i> ) (whole fish) .. .. .	Trace only	..
Sardine fish oil, 3 years old (whole fish) .. .. .	2.50	..
Beef liver .. .. .	131.00	..
„ muscle .. .. .	0.23	Trace only
Sheep liver .. .. .	87.00	..
„ muscle (mutton) .. .. .	0.12	Trace only
Butter (from local dairy) .. .. .	14.60	0.80
Ghee ( „ „ „ ) .. .. .	10.40	Nil.
Skim-milk powder (New Zealand) .. .. .	Nil	Nil.
Hen's egg (English poultry) (yolk only) .. .. .	15.00	32.60
„ „ (country „ ) ( „ „ ) .. .. .	12.70	28.40

TABLE II—*concl'd.*

Material.	Vitamin-A content per gramme ( $\gamma$ ).	Carotene content per gramme ( $\gamma$ ).
Crab (caught from the local hill streams) ..	Trace only	13.00
Chasker fish* (flesh only) (from West Coast) ..	Trace only	0.14
Aila fish* ( <i>Scomber microlepidotus</i> ) (flesh only) (from West Coast).	0.25	0.10
Keelakkan fish* ( <i>Sillago sihama</i> ) (flesh only) (from West Coast).	Trace only	0.10
Keluthi fish* (cat fish) (flesh only) (from West Coast)	Trace only	Trace only
Madava* (Mugil) fish ( „ ) ( „ „ „ )	0.10	0.20
Prawn „ ( „ ) ( „ „ „ )	Trace only	Trace only
Sorra fish* ( <i>Carcharias</i> ) (flesh only) (from West Coast)	0.20	0.10
Panna „ * ( <i>Ottolithus</i> sp.) ( „ ) ( „ „ „ )	Trace only	Trace only

\* These are Tamil or Malayalee words. For chasker the scientific name could not be traced.

That carotene may exist in different isomeric states ( $\alpha$ ,  $\beta$ , and  $\gamma$  forms, the second being about twice as potent as either the first or third) was not considered; only the total carotene content was estimated. One  $\gamma$  (0.001 mg.) of these carotene figures will correspond to about 1 International Unit, which is equal to 0.6 $\gamma$  of pure  $\beta$  carotene. One  $\gamma$  of the vitamin-A data = 2.6 I. U. (De, 1937).

TABLE III.

*The carotene content of some fruits and vegetable foods.*

Material.	Carotene content per gramme ( $\gamma$ ).
FRUITS.—	
Dates (Persian, preserved) .. ..	6.0
Guava (hill variety), ripe .. ..	Trace only
„ (country) „ .. ..	Trace only
Jack-fruit (ripe) .. ..	5.4
Mango, Ankola (from Bombay) .. ..	18.0
„ „ (after further ripening for 1 week due to keeping in cold storage).	24.4



TABLE III—*contd.*

Material.	Carotene content per gramme ( $\gamma$ ).
<b>FRUITS—<i>concl'd.</i></b>	
Mango, Ankola (after further ripening for 2 weeks due to keeping in cold storage).	32.0
„ „ Alphonso (from Bombay) ..	25.0
„ „ (after further ripening for 1 week due to keeping in cold storage).	32.0
„ South Indian (Nilam), ripe ..	61.6
„ „ „ (Kotaiyam), ripe ..	54.0
„ „ „ (Mysore Gundoo), ripe ..	51.2
„ „ „ (Salem graft) „ ..	48.0
„ „ „ (Malgoa) „ ..	28.0
„ „ „ (Kotagiri) „ ..	23.3
„ „ „ (Salem village) „ ..	22.5
„ „ „ (Malabar) „ ..	16.0
„ „ „ ( „ ), partly ripe ..	3.9
Plums (yellow variety) .. ..	2.3
„ (white skin) .. ..	Trace only
Pineapple (tender) .. ..	<i>Nil</i> or trace
„ (ripe) .. ..	0.6
<b>VEGETABLES : MISCELLANEOUS.—</b>	
Red-palm oil (Burma) .. ..	569.0
„ „ (Malaya) .. ..	520.0
Drumstick leaves ( <i>Moringa oleifera</i> ) ..	113.3
Fenugreek leaves ( <i>Trigonella fœnum-græcum</i> ) ..	38.6
Ipomœa ( <i>Ipomœa reptans</i> ) .. ..	32.7
Mint ( <i>Mentha viridis</i> ) .. ..	27.0
Sandakai ( <i>Solanum torvum</i> ), dry .. ..	7.5
Brinjal, thorny ( <i>Solanum xanthocarpum</i> ) ..	4.8
Ginger, new fresh .. ..	4.0

TABLE II—*concl'd.*

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Aila fish* ( <i>Scomber microlepidotus</i> ) (flesh only) (from West Coast).	0.25	0.10
Keelakkan fish* ( <i>Sillago sihama</i> ) (flesh only) (from West Coast).	Trace only	0.10
Keluthi fish* (cat fish) (flesh only) (from West Coast)	Trace only	Trace only
Madava* (Mugil) fish ( .. ) ( .. " " )	0.10	0.20
Prawn .. ( .. ) ( .. " " )	Trace only	Trace only
Sorra fish* ( <i>Carcharias</i> ) (flesh only) (from West Coast)	0.20	0.10
Panna .. * ( <i>Ottolithus</i> sp.) ( .. ) ( .. " " )	Trace only	Trace only

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That carotene may exist in different isomeric states ( $\alpha$ ,  $\beta$ , and  $\gamma$  forms, the second being about twice as potent as either the first or third) was not considered; only the total carotene content was estimated. One  $\gamma$  (0.001 mg.) of these carotene figures will correspond to about 1 International Unit, which is equal to 0.6 $\gamma$  of pure  $\beta$  carotene. One  $\gamma$  of the vitamin-A data = 2.6 I. U. (De, 1937).

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Mango, Ankola (from Bombay) .. ..	18.6
.. .. (after further ripening for 1 week due to keeping in cold storage).	24.4

in extracts from biological materials. Baumann and Steenbock (1933), MacWalter (1934), Nøtveid (loc. cit.) and other workers have studied the action of oxidation, heat, and irradiation on several vitamin-A concentrates. They report that in effecting selective destruction of vitamin A, ultra-violet irradiation gave satisfactory results, while the other two agents caused marked increase of absorption in the shorter ultra-violet and the products also showed absorption at 3,280 Å. U. In the course of the present study, extracts (concentrated) of the unsaponifiable fractions of several vitamin-A-free oils (gingelly oil, sardine fish oil, Chamban fish oil, etc.) were directly spectrographed. They all showed appreciable degrees of absorption in the region 3,700 Å. U. to 3,000 Å. U. The absorption increased very rapidly towards the shorter ultra-violet, while in the visible region it was absent or negligible. On irradiation, a slightly increased absorption was often noted in the shorter regions with little or no effect (increase or decrease of absorption) above 3,000 Å. U.

These observations show that irradiation for a reasonable period does not materially influence the irrelevant absorptions at 3,280 Å. U. and 4,500 Å. U. to 4,600 Å. U. The effect of irradiation on these vitamin-A-free oils direct was, however, found to be uncertain, since it sometimes caused an increased absorption throughout the whole of the ultra-violet. In the case of very potent oils (cod-liver oil, Haliverol, etc.) no such difficulty was experienced.

The gross intensity of absorption at 3,280 Å. U. and at 4,500 Å. U. to 4,600 Å. U. is in part due to vitamin A and carotene respectively and in part due to foreign materials which exhibit absorption, not necessarily selective in the regions concerned. In order to estimate the correct value of absorption due to vitamin A or carotene, it is necessary to remove either the foreign materials or the factor under test. Complete removal of impurities by chemical means and by subjection to very low temperature, etc., is an extremely difficult task and still remains uncertain. Strain (1935) reports that even carefully prepared solutions of carotene may contain various colourless impurities. In the present state of knowledge, ultra-violet irradiation, on preferably the unsaponifiable fractions of the poor and moderately rich materials, appears to be a very convenient and satisfactory method of removing vitamin A and carotene selectively. Determination of the difference between the absorptions before and after irradiation will thus give a correct measure of the potent factor present. The method has been applied in the analysis of very potent materials, as well as very poor ones, potency ranging from 13,000 $\gamma$  to 0.1 $\gamma$  per gramme. The results are quite satisfactory and encouraging.

From Tables II and III, it is seen that, with the exception of liver, liver oils, butter, ghee, and egg-yolk, flesh foods in general are very poor in vitamin A. Green leafy vegetables, ripe fruits such as mangoes, oranges, tomatoes, papaya, etc. (De, 1936a), are particularly rich in carotene; their carotene content in most cases approaches or exceeds the vitamin-A activity (carotene and vitamin A *strictu sensu*) of egg-yolk or milk fats. Red-palm oil stands pre-eminent in the list of vegetable foodstuffs and its potency is comparable to that of an average sample of medicinal cod-liver oil. From the results of the assays of ripe and green fruits, it appears that vitamin-A potency (carotene content) increases during ripening. The carotene content of the fruits analysed was found to be proportional to the degree of ripeness and the intensity of the yellow coloration.

Biological experiments by Morgan and Smith (1928), House *et al.* (1929), and other workers on tomatoes, ripened both artificially and naturally, resulted in similar conclusions.

Various samples of medicinal cod-liver oil (Norwegian) analysed showed wide differences in potency, vitamin-A content ranging from 150 $\gamma$  to 600 $\gamma$  per gramme. This is quite possible, since the vitamin-A content of such oils may vary according to the age, diet, and source of the fish caught; and a deterioration may also occur which is due to mere storage for prolonged periods. It is strange to note that the sample of giant-ray-liver oil was devoid of vitamin A. The lay public goes simply by the name and never questions the potency of an oil they are advised to take. It would be of public health value, if manufacturers were obliged to state what are the original potencies of their products and to what extent these are maintained under the conditions of filling and storage.

### SUMMARY.

1. Experiments were made to study the partition of vitamin A from petrol to alcohol. For a quantitative partition saponification was found to be necessary, and as many as 7 to 10 washings were required. The method applied to unsaponifiable fractions was equally effective in the reverse direction, namely, from alcohol to petrol. It appears that the presence of foreign materials having unequal solubilities in the two solvents affects the partition co-efficient of vitamin A between the two phases.

2. Vitamin-A solutions could be freed from various interfering pigments by adsorption on charcoal without causing appreciable loss. This procedure, with due precautions, was found to be fairly satisfactory for use in routine work.

3. Solutions of vitamin A and carotene in different solvents were irradiated and their stabilities to light compared. Experiments were made to find out the conditions, viz., solvent, concentration, the time of exposure, etc., that are most suitable for spectrophotometric studies. Both vitamin A and carotene are highly unstable in chloroform. Convincing reasons are given why use of chloroform should be avoided in spectrographic work.

4. Considerable evidence was obtained in support of the validity of the technique. It is concluded that the difference in absorption before and after irradiation gives a very accurate measure of the vitamin A or carotene present. This principle applied, on preferably the unsaponifiable fractions of poor and moderately rich materials, eliminates any absorption due to impurities.

5. The vitamin-A and carotene contents of some 70 additional foodstuffs have been assayed using the modified and improved technique as described.

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THE ASSIMILATION OF VITAMIN A AND CAROTENE BY  
RATS FROM SOME COMMON FOODS WITH A NOTE ON  
THE CONVERSION FACTOR I. U./E., PROPOSED  
BY THE INTERNATIONAL VITAMIN  
CONFERENCE.

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IN previous communications (De, 1936, 1937) it has been pointed out that the vitamin-A potency (as indicated by the carotene content) of many vegetable foodstuffs, particularly green leafy vegetables, and ripe fruits such as mango, papaya, orange, tomato, etc., may approach or even exceed that of egg-yolk and milk fat. A few samples of red-palm oil tested have been found to be very rich in carotene, and their carotene values indicate that these oils are as potent as an average sample of medicinal cod-liver oil. Hence carotene-rich vegetable foods, which in general are much cheaper and more easily available than animal products, appear to be of great importance as a source of vitamin-A activity. Although it might be expected that vitamin A from animal matter would be better utilized than its precursor in vegetable foods, we have so far no precise knowledge on this question.

Vegetable foods, because of their cellular structure, are in general less digestible than animal foods. The faeces, particularly those of young children, are frequently found to contain undigested materials derived from green vegetables and fruits. It seems probable that both vitamins and minerals must be lost through incomplete digestion, and hence the nutrients of vegetable foods would be less efficiently utilized. That vitamin A and carotene may be lost through incomplete digestion has been reported by several workers (Rowntree, 1930; Moore, 1931; Baumann and Steenbock, 1934; and others). The loss invariably occurred by excretion in the faeces; the urine was observed to be always devoid of vitamin A and carotene. The greatest loss as well as the greatest retention occurred with the greatest intake. Moore (1931) detected the presence of unchanged carotene throughout the alimentary tract (of rats), and the pigmented fat derived from the faeces was found to be biologically active, at a level corresponding to its apparent carotene content. Baumann and Steenbock (1934) report that only about 5 to 10 per cent of vitamin A ingested by vitamin-A-depleted rats

in a single dose of 170 to 1,700 International Units (I. U.) could be recovered in the liver; no storage in the liver resulted on feeding 34 I. U. Faecal excretion of vitamin A could account for only a small fraction, while the major portion was destroyed in the digestive tract. They report also that there is a parallelism between the absorption of vitamin A occurring in rats and that occurring in children as observed by Clausen (1933). Rowntree (*loc. cit.*) has found that vitamin A is not excreted in the urine of infants and young children, even when generous amounts are fed; without exception, faecal loss was noted varying from 2 to 12 per cent of the intake, and being apparently proportional to the loss of fat.

It has been observed that the biological activity of carotene depends on the nature of the solvent. The use of mineral oils as solvent considerably hinders the absorption of carotene, as shown by the recovery of carotene from the faeces (Dutcher *et al.*, 1934). So also different biological values for pure carotene may be obtained by the use of different vegetable oils as solvent, or by different samples of the same solvent (Baumann and Steenbock, 1933; Culhane *et al.*, 1934; and other workers). There is no very definite evidence as to stability of carotene in these tests, and it is impossible to say whether the difference is due to variation in the utilization or variation in the stability of the carotene.

Ahmad (1931) reports that the absorption of carotene (in rats), fed in ethyl-laurate solution, was considerably affected by the absence of fat in the diet. When carotene was fed in red-palm oil, in conjunction with fat-poor and fat-free diets, about the same amount—40 per cent of the carotene ingested—was recovered from the faeces. Greaves and Schmidt (1935) report that the fat content of a diet, within the limits of 3 to 20 per cent, does not materially influence the absorption of carotene when the latter is fed in cotton-seed oil, at a level of 5 $\gamma$  to 15 $\gamma$  per rat per day.

From the literature, it appears that there has so far been very little work done in this field. Accordingly, an investigation was undertaken to study the relative utilization of vitamin A and carotene by rats from a number of natural foods. The present work throws some light on this point, which is of obvious importance. Simultaneously, experiments were carried out to study how the difference in body-weights of the experimental animals, and the fat, vitamin B and the mineral content of the diet may influence the absorption of vitamin A and carotene.

#### EXPERIMENTAL.

Several groups of stock rats were kept on a vitamin-A-free basal diet for 7 to 12 days, until the faeces were free from vitamin A and carotene. The animals were then given diets containing known amounts of vitamin A or carotene. The total amount of vitamin A or carotene lost by excretion in the faeces was determined. The difference between the amounts ingested and excreted was taken as a measure of the amount absorbed or retained in the body. Absorption or retention, in this sense, includes the amounts of vitamin A or carotene that are destroyed in the digestive tract. No loss was ever found to occur through the urine. The test materials were fed to groups of rats, for periods ranging from 3 to 5 days; in some cases absorption from a single feeding of the test substance was studied. The following materials were fed as sources of vitamin A or carotene: halibut-liver



oil, cod-liver oil, butter, egg-yolk, amaranth, papaya, orange juice, red-palm oil, and two mixed diets. The first, second, third, sixth, seventh, and eighth named materials were fed separately, before the main diets were supplied. Amaranth and egg-yolk were first mixed with small amounts of the vitamin-A-free synthetic diet, and fed to the animals before the bulk of the main diet was supplied. The carotene containing mixed diets was supplied in liberal quantities (known amounts), and the amount consumed calculated by weighing out the residue, loss of weight due to evaporation of moisture being accounted for.

The composition of the basal diet and the two mixed diets was as follows :—

<i>Basal diet.</i>	Parts.
Starch .. .. .	63
Casein .. .. .	20
Coco-nut oil .. .. .	10
Salt mixture .. .. .	5
Yeast .. .. .	2
With tap water to drink	

<i>Mixed diet No. 1.</i>	Parts.
Cholam ( <i>Sorghum vulgare</i> ) .. .. .	2·00
Red gram ( <i>Cajanus indicus</i> ) .. .. .	0·25
Ground-nut ( <i>Arachis hypogea</i> ) .. .. .	0·06
Amaranth ( <i>Amaranthus gangeticus</i> ) .. .. .	0·01
Brinjal ( <i>Solanum melongena</i> ) .. .. .	0·01
Bitter gourd ( <i>Momordica charantia</i> ) .. .. .	0·01
Common salt .. .. .	0·06
With tap water to drink	

<i>Mixed diet No. 2.</i>	Parts.
Raw polished rice .. .. .	12·0
Cholam ( <i>Sorghum vulgare</i> ) .. .. .	6·0
Red gram ( <i>Cajanus indicus</i> ) .. .. .	1·0
Root vegetables .. .. .	2·0
Leafy vegetables .. .. .	8·0
Ground-nut ( <i>Arachis hypogea</i> ) .. .. .	1·0
Soya bean ( <i>Glycine hispida</i> ) .. .. .	1·0
Coco-nut oil .. .. .	1·0
Butter-milk .. .. .	10·0
Dried skim milk (New Zealand) .. .. .	0·5
Jaggery .. .. .	1·0
With tap water to drink	

This diet was devoid of vitamin A : no vitamin A was detected in the butter-milk used.

Synthetic diets, other than the basal diet, which were free from fat, yeast (vitamin-B complex), or salt mixture, were prepared by substituting starch for the factor eliminated from the basal diet. The mixed diet No. 1 was based on a diet given to children in famine camps in the Madras Presidency. Aykroyd and Krishnan (1936a) observed that a high percentage of the children consuming this

diet, supplying roughly 700 $\gamma$  of carotene daily, showed symptoms of vitamin-A deficiency. It was felt to be of interest to determine carotene absorption from this diet. Mixed diet No. 2 is one of the 'cheap balanced diets' devised and investigated in this laboratory (Aykroyd and Krishnan, 1936b).

The results of the various experiments are shown in Tables I, II, and III, which supply details about the number of animals used, period of feeding, amount of vitamin A and carotene fed and the amount excreted in the faeces, etc. The investigation includes studies of (1) the absorption of vitamin A and carotene by rats from a number of natural foods and (2) the influence of several factors on the absorption of vitamin A and carotene. These were: body-weight of the experimental animals, level of vitamin-A or carotene intake, and the fat, yeast (vitamin-B complex), and mineral content of the diet.

The estimation of vitamin A and carotene in the various foods, as well as in the faeces, was carried out by the modified spectrophotometric method (De, 1935, 1937). Carotene from the various test materials was extracted directly, while vitamin A in general was extracted from the unsaponifiable fraction; in the case of halibut-liver oil and cod-liver oil, test solutions were directly prepared.

TABLE I.

1. *The absorption of vitamin A from butter (fat-rich diet).*

Days.	Quantity of vitamin A fed daily to the whole group of rats ( $\gamma$ ) (0.001 mg.).	Quantity of vitamin A excreted in faeces ( $\gamma$ ).	Total quantity of vitamin A fed ( $\gamma$ ).	Total quantity of vitamin A excreted ( $\gamma$ ).	Percentage of vitamin A excreted.	Percentage of vitamin A retained in the body (from columns 4 and 5).
(1)	(2)	(3)	(4)	(5)	(6)	(7)
1	105.6	2.5	542.4	17.8	3.3	96.7
2	105.6	3.8				
3	105.6	4.7				
4	112.8	3.7				
5	112.8	..				
6	0.0	3.1				
7	0.0	0.0				
8	0.0	0.0				

The group consisted of 6 rats of body-weights ranging from 130 g. to 170 g. They were fed on the basal diet with an addition of 2 g. of butter per rat per day. The diet including butter contained about 30 to 40 per cent of fat. The quantity of vitamin-A intake was about 18 $\gamma$  per rat per day. The presence of a small

quantity of carotene in butter was disregarded. The fæces of the 5th and 6th days were tested together.

TABLE I—*concl'd.*2. *The absorption of vitamin A from cod-liver oil (fat-free diet).*

Days.	Quantity of vitamin A fed daily to the whole group of rats ( $\gamma$ ) (0.001 mg.).	Quantity of vitamin A excreted in fæces ( $\gamma$ ).	Total quantity of vitamin A fed ( $\gamma$ ).	Total quantity of vitamin A excreted ( $\gamma$ ).	Percentage of vitamin A excreted.	Percentage of vitamin A retained in the body (from columns 4 and 5).
(1)	(2)	(3)	(4)	(5)	(6)	(7)
1	82.2	..	411.0	19.2	4.7	95.3
2	82.2	..				
3	82.2	..				
4	82.2	14.2				
5	82.2	..	5.0	..	..	..
6	0.0	..				
7	0.0	5.0				
8	0.0	0.0				

The group consisted of 6 rats of body-weights ranging from 130 g. to 160 g. They were fed by hand with 1 drop of cod-liver oil per rat per day. The fat content of the basal diet was replaced by starch and sufficient quantities of the fat-free diet were supplied to the animals. The level of vitamin-A intake per rat per day was about 14.0 $\gamma$ . The fæces of the first 4 days and of 5th, 6th, and 7th days were tested together.

TABLE II.

1. *The absorption of carotene from amaranth (large supply of carotene).*

Days.	Quantity of carotene fed daily to the whole group of rats ( $\gamma$ ) (0.001 mg.).	Quantity of carotene excreted through the fæces ( $\gamma$ ).	Total quantity of carotene fed ( $\gamma$ ).	Total quantity of carotene excreted ( $\gamma$ ).	Percentage of carotene excreted.	Percentage of carotene retained in the body (from columns 4 and 5).
(1)	(2)	(3)	(4)	(5)	(6)	(7)
1	800.0	137.0	1,752.0	1,004.0 + roughly 30 per cent of 1,004.0 = 1,305.0	74.0 (roughly)	26.0 (roughly)
2	576.0	372.0				
3	576.0	495.0				

The group consisted of 3 rats of body-weights ranging from 120 g. to 160 g. They were fed on the basal diet including 2 g. of fresh amaranth leaves per rat per day. The level of carotene intake per rat per day was about 195·0 $\gamma$ . Examination of the faeces for carotene after the supply was stopped was not carried out. Experience showed that carotene excretion may continue for some days after the removal of a supply from the diet and 20 to 40 per cent of the total carotene excretion may occur during this time.

TABLE II—*contd.*2. *The absorption of carotene from amaranth (small supply of carotene).*

Days.	Quantity of carotene fed daily to the whole group of rats ( $\gamma$ ) (0·001 mg.).	Quantity of carotene excreted through the faeces ( $\gamma$ ).	Total quantity of carotene fed ( $\gamma$ ).	Total quantity of carotene excreted ( $\gamma$ ).	Percentage of carotene excreted.	Percentage of carotene retained in the body (from columns 4 and 5).
(1)	(2)	(3)	(4)	(5)	(6)	(7)
1	72·0	15·0	381·0	207·0	54·3	45·7
2	77·0	60·0				
3	77·0	22·0				
4	88·0	36·0				
5	67·0	30·0				
6	0·0	18·0				
7	0·0	14·0				
8	0·0	8·0				
9	0·0	4·0				
10	0·0	0·0				

The group consisted of 6 rats of body-weights ranging from 100 g. to 140 g. They were fed on the basal diet including about 0.15 g. of fresh amaranth per rat per day. The level of carotene intake was about 13.0 $\gamma$  per rat per day.

TABLE II—*contd.*3. *The absorption of carotene from amaranth (fat-rich diet).*

Days.	Quantity of carotene fed daily to the whole group of rats ( $\gamma$ ) (0.001 mg.).	Quantity of carotene excreted through the faeces ( $\gamma$ ).	Total quantity of carotene fed ( $\gamma$ ).	Total quantity of carotene excreted ( $\gamma$ ).	Percentage of carotene excreted.	Percentage of carotene retained in the body (from columns 4 and 5).
(1)	(2)	(3)	(4)	(5)	(6)	(7)
1	120.0	38.3	564.0	199.0	35.3	64.7
2	120.0	22.0				
3	120.0	28.6				
4	96.0	30.0				
5	108.0	24.2				
6	0.0	20.0				
7	0.0	12.3				
8	0.0	11.0				
9	0.0	7.6				
10	0.0	5.0				
11	0.0	0.0				
12	0.0	0.0				

The group consisted of 6 rats of body-weights ranging from 130 g. to 180 g. They were given a small quantity of amaranth + basal diet + 2 g. of coco-nut oil per rat per day. The fat content of the diet was about 30 to 40 per cent. The level of carotene intake was about 19.0 $\gamma$  per rat per day.

TABLE II—*contd.*4. *The absorption of carotene from a mixed vegetable diet (diet No. 1) in groups of rats of different body-weights.*

Description.	Days.	Quantity of carotene fed daily to the whole group of rats ( $\gamma$ ) (0.001 mg.).	Quantity of carotene excreted through the faeces ( $\gamma$ ).	Total quantity of carotene fed ( $\gamma$ ).	Total quantity of carotene excreted ( $\gamma$ ).	Percentage of carotene excreted.	Percentage of carotene retained in the body (from columns 4 and 5).
	(1)	(2)	(3)	(4)	(5)	(6)	(7)
Group I: 3 rats, body-weights 40 g. to 50 g. Level of carotene intake about 13.0 $\gamma$ per rat per day.	1	51.0	16.7	191.0	94.3	49.4	50.6
	2	23.0	19.0				
	3	39.0	21.0				
	4	39.0	12.6				
	5	39.0	10.0				
	6	0.0	6.0				
	7	0.0	5.0				
	8	0.0	..				
	9	0.0	4.0				
	10	0.0	0.0				
Group II: 3 rats, body-weights 100 g. to 120 g. Level of carotene intake about 19.0 $\gamma$ per rat per day.	1	76.5	22.0	289.5	137.6	47.5	52.5
	2	39.0	43.0				
	3	58.0	20.6				
	4	58.0	17.0				
	5	58.0	10.0				
	6	0.0	8.0				
	7	0.0	10.0				
	8	0.0	..				
	9	0.0	7.0				
	10	0.0	0.0				

The higher intake in Group II was due to the consumption of larger quantities of food.

TABLE II—*contd.*5. *The absorption of carotene from red-palm oil (fat-free diet).*

Days.	Quantity of carotene fed daily to the whole group of rats ( $\gamma$ ) (0.001 mg.).	Quantity of carotene excreted through the faeces ( $\gamma$ ).	Total quantity of carotene fed ( $\gamma$ ).	Total quantity of carotene excreted ( $\gamma$ ).	Percentage of carotene excreted.	Percentage of carotene retained in the body (from columns 4 and 5).
(1)	(2)	(3)	(4)	(5)	(6)	(7)
1	78.2	16.5	330.8	136.8	41.4	58.6
2	96.0	30.0				
3	64.2	28.0				
4	54.0	24.3				
5	38.4	18.0				
6	0.0	9.5				
7	0.0	..				
8	0.0	6.5				
9	0.0	4.0				
10	0.0	0.0				

The group consisted of 6 rats of body-weights ranging from 110 g. to 140 g. They were supplied with sufficient quantities of fat-free basal diet + 1 drop of

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red-palm oil per rat per day (fed by hand). The level of carotene intake was about 11.0 $\gamma$  per rat per day. The faeces of 7th and 8th days were extracted together.

TABLE II—concl'd.

6. *The absorption of carotene from red-palm oil in groups of rats of different body-weights.*

Description.	Days.	Quantity of carotene fed daily to the whole group of rats ( $\gamma$ ) (0.001 mg.).	Quantity of carotene excreted through the faeces ( $\gamma$ ).	Total quantity of carotene fed ( $\gamma$ ).	Total quantity of carotene excreted ( $\gamma$ ).	Percentage of carotene excreted.	Percentage of carotene retained in the body (from columns 4 and 5).
	(1)	(2)	(3)	(4)	(5)	(6)	(7)
Group I: 3 rats body-weights 40 g. to 50 g. Fed with a single dose of carotene = 117.0 $\gamma$ per rat.	1	351.0	108.0	351.0	150.0	42.7	57.3
	2	0.0	29.0				
	3	0.0	10.0				
	4	0.0	3.0				
	5	0.0	0.0				
	6	0.0	0.0				
	7	0.0	0.0				
Group II: 3 rats, body-weights 100 g. to 120 g. Fed with a single dose of carotene = 117.0 $\gamma$ per rat.	1	351.0	96.0	351.0	155.0	44.2	55.8
	2	0.0	39.0				
	3	0.0	13.0				
	4	0.0	5.0				
	6	0.0	2.0				
	7	0.0	0.0				
	8	0.0	0.0				



TABLE III.  
*The absorption of vitamin A and carotene from various foods.*

Experiment number.	Nature of the food and the method of supply.	Number of experimental rats.	Daily dose of vitamin A and carotene per rat.	Total quantity of vitamin A or carotene fed to the whole group (γ).	Total quantity of vitamin A or carotene excreted by the whole group (γ).	Percentage of vitamin A or carotene excreted.	Percentage of vitamin A or carotene retained in the body (from columns 5 and 6).
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)
1	Basal diet without yeast (deficient in vitamin-B complex), red-palm oil fed by hand in a single dose.	3	120.0	360.0 carotene.	154.5 carotene.	43.0 carotene.	57.0 carotene.
2	Basal diet with red-palm oil fed by hand in a single dose.	3	120.0	360.0 "	164.0 "	45.6 "	54.4 "
3	Red-palm oil fed by hand in a single dose. Basal diet with 2 g. of coco-nut oil per rat per day.	2	60.0	180.0 "	70.0 "	38.9 "	61.1 "
4	Red-palm oil fed by hand in a single dose. Basal diet without salt mixture.	3	120.0	360.0 "	167.0 "	46.4 "	53.6 "
5	Hallibut-liver oil fed by hand in a single dose, basal diet.	3	230.0	690.0 vitamin A.	26.2 vitamin A.	3.8 vitamin A.	96.2 vitamin A.

TABLE III—*concl'd.*

Experiment number.	Nature of the food and the method of supply.	Number of experimental rats.	Daily dose of vitamin A and carotene ( $\gamma$ ) per rat.	Total quantity of vitamin A or carotene fed to the whole group ( $\gamma$ ).	Total quantity of vitamin A or carotene excreted by the whole group ( $\gamma$ ).	Percentage of vitamin A or carotene excreted.	Percentage of vitamin A or carotene retained in the body (from columns 5 and 6).
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)
6	Egg-yolk mixed with basal diet; fed for 3 days.	6	17.0 6.0	306.0 carotene. 108.0 vitamin A.	118.0 carotene. 3.0 vitamin A.	38.6 carotene. 2.8 vitamin A.	61.4 carotene. 97.2 vitamin A.
7	Amaranth mixed with basal diet without fat; fed for 3 days.	6	32.5	585.0 carotene.	301.0 carotene.	51.5 carotene.	48.5 carotene.
8	Mixed diet No. 2; fed for 3 days.	6	60.0	1,080.0 "	435.0 "	40.3 "	59.7 "
9	Papaya mixed with basal diet; fed in a single dose.	8	26.0	208.0 "	90.0 "	43.3 "	56.7 "
10	Basal diet + orange juice fed by hand in a single dose.	8	10.0	80.0 "	33.0 "	41.2 "	58.8 "
11	Basal diet with very small amount of carotene; fed by hand in coco-nut oil in a single dose.	16	1.0	16.0 "	6.0 "	37.5 "	62.5 "

The feces passed during 5 days in the experiments Nos. 1, 2, 3, 4, 5, 9, 10, and 11 and during 8 days in the experiments Nos. 6, 7; and 8, after the first feeding, were collected together and examined as a single test material.

## DISCUSSION.

From Tables I and II, it is seen that excretion of carotene in the faeces continued for 4 to 5 days, while that of vitamin A ceased within 2 to 3 days, after the supply of carotene or vitamin A respectively was stopped. Vitamin A was not detected in the faeces of carotene-fed animals. It was also observed that during the period in which vitamin-A depletion of the liver of rats was taking place, no faecal excretion of vitamin A occurred. It follows that faecal loss of vitamin A and carotene is due merely to incomplete digestion; the liver stores do not contribute to the faecal excretion. Depletion of the vitamin-A reserve, when the diet is deficient in vitamin-A activity, must be due solely to oxidation or some chemical transformations occurring within the tissues.

The data set out in Tables I and III (columns 5 and 6) show that only very little, 3 to 5 per cent, of vitamin A ingested (contained in halibut-liver oil, cod-liver oil, butter, and egg-yolk) was recovered from the faeces when the animals were fed at a level of  $6\gamma$  to  $230\gamma$  per rat per day. Baumann and Steenbock (1934) found that only a small fraction of vitamin A ingested could be recovered from the liver and faeces. They conclude that the major portion of vitamin A ingested is destroyed in the digestive tract. It is probable that some part of the unexcreted carotene will also be destroyed in the intestinal tract; precise knowledge on this question is, however, still lacking.

Excretion of carotene fed in amaranth, orange juice, papaya, egg-yolk, and the mixed diets, under varying conditions, ranged from 39 to 55 per cent (average 46 per cent) of the intake (Tables II and III), when fed at the daily levels of  $10\gamma$  to  $60\gamma$ . The faecal loss of carotene was found to be considerably greater when carotene was fed (in amaranth) in relatively large amounts—about  $200\gamma$  per rat per day. Excretion of carotene when fed in red-palm oil, under varying conditions (Tables II and III), ranged from 37 to 47 per cent (average 42.5 per cent), when supplied at a level of  $1\gamma$  to  $120\gamma$  per rat per day. It is interesting to note that complete absorption of carotene never occurred, and even at the minimum level ( $1.0\gamma$ ) of intake, 37.5 per cent was found to be excreted in the faeces. From Table II (column 1) and Table III (columns 1, 2, and 4) it appears that large amounts of carotene are probably better utilized when fed in oil solutions than when contained in natural foods, such as amaranth. The fat content of a diet, within the limits of 0 to 40 per cent, did not cause any significant difference in the absorption of vitamin A and carotene. An exception to this was noted in one case—Table II (column 3): absorption of carotene from amaranth was increased by about 10 per cent when the amaranth was fed with the basal diet containing about 30 to 40 per cent of fat.

Experiments Nos. 1, 2, 3, and 4, Table III, show that the absence of the vitamin-B complex (yeast) and the salt mixture, from a diet, does not materially influence the retention of carotene; so also retention of carotene was unaffected (Table II, columns 5 and 6) by the difference in body-weights of the experimental animals.

The above observations refer only to healthy and normal animals. The possibility, however, remains that absorption of vitamin A and carotene may be affected by various factors in the presence of pathological symptoms. Recently,

there have been several reports (Ralli *et al.*, 1935 ; Heymann, 1936*a* and *b* ; Wendt, 1936 ; and other workers), which show that the metabolism of both vitamin A and carotene may be affected in the absence of bile, in diabetes, hyperthyroidism, and in various infectious diseases.

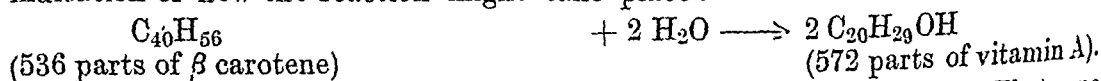
It may be noted that in the light of Clausen's (*loc. cit.*), and Baumann and Steenbock's (1934) work, the results of the present experiment on rats may be applicable to human beings.

#### A NOTE ON THE CONVERSION FACTOR I. U./E.

Carr and Jewell (1933) and Karrer and Morf (1933) have been able to isolate small quantities of vitamin A, in very pure forms, and their preparations showed  $E \frac{1 \text{ per cent}}{1 \text{ cm.}}$  3,280 A. U.=1,600 and 1,700 respectively. With the help of this data, spectrophotometric assays of vitamin A have become quantitative and the vitamin-A content of a test material can be expressed in absolute quantities. It is, however, desirable that vitamin-A values, estimated in absolute quantities, should be convertible into the customary unit, namely, the International Unit of vitamin A, which has been defined as the vitamin-A activity of 0.6 $\gamma$  of pure  $\beta$  carotene. With a view to discovering the value of the conversion factor (I. U./E.), simultaneous biological and spectrophotometric studies were carried out, in different laboratories, on several given samples of cod-liver oil (Medical Research Council, 1935). The results varied widely, between 750 to 2,380.

The Vitamin-A Sub-Committee and the International Vitamin Conference, League of Nations, Health Organization (1934), on scrutinizing all the available data, have recommended the figure I. U./E.= 1,600 to be adopted provisionally, as a means of converting spectrographic values into International Units of vitamin A. From the figures, viz.,  $E \frac{1 \text{ per cent}}{1 \text{ cm.}} = 1,600$  or 1,700 for pure vitamin A, and I. U./E.= 1,600, it follows that one gramme of pure vitamin A will contain 256 to 272,000 I. U. ; or, in other words, 0.37 $\gamma$  to 0.39 $\gamma$  of vitamin A is equivalent to one I. U. (0.6 $\gamma$  of pure  $\beta$  carotene). It thus appears that pure vitamin A is about 1½ times more potent than pure  $\beta$  carotene, a deduction contrary to theoretical considerations.

In the *in vivo* transformation of carotene into vitamin A, the following is an indication of how the reaction might take place :—



Therefore 1 $\gamma$  of  $\beta$  carotene is converted into 1.067 $\gamma$  of vitamin A. That one molecule of  $\beta$  carotene may be converted into two molecules of vitamin A is substantiated by the fact that, according to the molecular structure, one molecule of  $\alpha$  carotene can give rise to only one molecule of vitamin A and the  $\alpha$  isomer is half as potent as the  $\beta$  form. Thus theoretically, equal quantities of vitamin A and  $\beta$  carotene should possess very nearly the same degree of biological potency. This truth has to some extent been experimentally realized by Moore (1933) and Morgan *et al.* (1935) from feeding experiments on rats.

The discrepancy between the relative biological values of vitamin A and carotene, as described in the previous paragraph, and the relative values deduced

from their chemical nature, has so far escaped attention. An explanation for this discrepancy can now be given. In the present study on the absorption of vitamin A and carotene in rats, it has been noted that faecal loss of vitamin A is negligible, while about 40 per cent of carotene ingested (in oil solution) is eliminated in the faeces. The faecal loss of carotene appears to be practically constant even when carotene is supplied at a minimum level of 1 $\gamma$  per rat per day. If we assume that the unexcreted fractions of vitamin A and carotene are equally affected by destruction taking place in the digestive tract, the following deductions are justified : out of 0.6 $\gamma$  of  $\beta$  carotene ingested,  $\frac{0.6 \times 40}{100} = 0.24\gamma$  will be excreted in the faeces, and 0.36 $\gamma$  will be retained in the body. As a result of *in vivo* transformation according to the reaction noted above, the unexcreted portion of  $\beta$  carotene, on absorption, will give rise to  $0.36 \times 1.067 = 0.38\gamma$  of vitamin A, an amount equal to that of vitamin A representing one I. U. as calculated above.

The factor I. U./E.=1,600, adopted provisionally, fits in quite harmoniously with the result of these absorption experiments and with the theoretical considerations, and hence, this value of the conversion factor appears to be the most appropriate. We can, therefore, conclude that the wide range of values (750 to 2,380), recorded for the ratio I. U./E., by various workers, is due rather to errors inherent in biological experiments than to any real variation in the ratio.

#### SUMMARY.

1. The relative utilization of vitamin A and carotene contained in a number of natural foods, namely, halibut-liver oil, cod-liver oil, butter, egg-yolk, red-palm oil, orange juice, papaya, amaranth, and two mixed diets, was investigated. The difference between the amount of vitamin A or carotene ingested in foods and that excreted in the faeces was taken as a measure of the amount absorbed in the body. 'Absorption', in this sense, includes the amounts of vitamin A or carotene that are destroyed in the digestive tract. Under varying conditions of feeding, absorption of vitamin A was found to be almost complete, while only 45 to 65 per cent of carotene ingested was retained in the body. It is thus clear that a considerable amount of carotene ingested is lost due to incomplete digestion and hence the biological value of vitamin A is much higher than that of its precursor, carotene.

2. The difference in the body-weights of experimental animals, and the fat, yeast (vitamin-B complex) and mineral content (salt mixture) of the diets did not cause any significant variation in the absorption of carotene. Vitamin A was found to be equally well absorbed from fat-free and fat-rich diets. It also appeared that large doses of carotene are better utilized when fed in an oil solution (red-palm oil) than in natural foods, such as amaranth.

3. The absorption experiments have provided some evidence in support of the appropriateness of the conversion factor I. U./E.=1,600, which was provisionally adopted by the International Vitamin Conference.

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## THE RELATIVE VALUE OF THE PROTEINS OF CERTAIN FOODSTUFFS IN NUTRITION.

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A SYSTEMATIC examination of the relative biological values of the proteins of foodstuffs was undertaken for reasons given below. The comparative biological value of the proteins of 13 foodstuffs, including 5 cereals—raw milled rice, whole wheat, cambu, cholam, and ragi; 3 pulses—lentil, green gram, and soya bean; and 5 vegetables—drumstick leaves, amaranth leaves, ipomœa leaves, lady's fingers, and brinjal, have been studied by the nitrogen-balance method.

The biological value of the proteins of foodstuffs and isolated proteins has been the subject of numerous investigations in the past. Reference may be made to the works of McCollum and Simmonds (1929), Mitchell (1924*a*), Mitchell and Hamilton (1929), and Boas-Fixsen (1935) which provide reviews of the voluminous literature. Study of the biological value of vegetable proteins has been largely confined to the proteins of cereals and pulses, and only recently has attention been given to the proteins of vegetables (Adolph *et al.*, 1935). Further, the relative biological value of the proteins of various cereals and pulses is still a matter of uncertainty as the available data are conflicting.

McCay (1912) investigated various Indian foodstuffs and worked out the 'co-efficient of absorption' of their proteins by deducting protein excreted in the faeces from the protein intake. He assigned a value of 70.3 for the total proteins of wheat, 85.6 for green gram, 49.4 for cambu, and 53.6 for cholam. From his long series of experiments on various other foodstuffs he concluded that 'it is a mere truism to state that the gross chemical value of a food material may not necessarily be a measure of its real nutritive value and that the real value depends on the degree of absorbability of its proximate principles'.

Later, McCollum and Simmonds (1917) found wheat, maize, and rice to be of equal value for the maintenance of body-weight in rats and inferior to oats and millet; but from the results of other long term experiments (McCollum and Simmonds, 1921), they arranged the cereal proteins in the following descending order of merit: wheat, barley, rye, maize, and oats. Osborne and Mendel (1918) found that rice and barley supported growth better than did oats and maize. Sherman and his co-workers (1918 to 1920) found wheat, maize, and oats of approximately equal value in supplying the maintenance requirements of adult human

beings. Neither Mitchell and Carman (1924, 1926) nor Boas-Fixsen and her co-workers (1932, 1934), using the balance-sheet method, found any difference between the biological value of the proteins of wheat and maize. Niyogi *et al.* (1932) reported a biological value of 58 for lentil and 60 for green gram, while Basu *et al.* (1936) obtained a value of 32 for lentil and 52 for green gram, the value (58) obtained for lentil by the former group of workers being nearly twice that (32) obtained by the latter group of workers, though the level of protein intake was the same in all these experiments.

#### MATERIALS USED AND THEIR COMPOSITION.

The cereals and pulses used in the experiments were ground to fine powder. In the case of vegetables, the edible portions were cut into small pieces, dried first in the open air and finally in an air oven at a temperature of 40°C. to 50°C., after which the dried materials were ground to fine powder. Drying was necessary in the case of vegetables, as in the fresh state vegetables contain about 90 per cent of water, while their protein content varies from 1.5 to 6 per cent. It was, therefore, impossible to use the fresh materials to prepare diets containing 5 per cent of vegetable protein without the bulk of the diet becoming too great. Moisture was determined by drying samples to constant weight in an air oven at 100°C. and the 'crude protein' content ( $N \times 6.25$ ) by the Kjeldahl method. The moisture and 'crude protein' content of the materials used as sources of protein are given in Table I:—

TABLE I.

*Moisture and 'crude protein' content of test materials.*

Name of foodstuff.	Botanical name.	Moisture per cent.	'Crude protein' per cent.
Raw milled rice .. ..	<i>Oryza sativa</i> .. ..	12.50	6.88
Whole wheat .. ..	<i>Triticum vulgare</i> .. ..	12.31	12.62
Cambu .. ..	<i>Pennisetum typhoideum</i> .. ..	12.10	10.48
Cholam .. ..	<i>Sorghum vulgare</i> .. ..	11.20	10.27
Ragi .. ..	<i>Eleusine coracana</i> .. ..	12.58	7.12
Lentil .. ..	<i>Lens esculenta</i> .. ..	12.44	25.70
Green gram .. ..	<i>Phaseolus radiatus</i> .. ..	10.42	23.80
Soya bean .. ..	<i>Glycine hispida</i> .. ..	9.30	40.00
Drumstick leaves (dry), powder (air dry).	<i>Moringa oleifera</i> .. ..	10.68	26.85
Drumstick „ (fresh) .. ..	„ .. ..	(75.00)	(6.65)
Amaranth leaves powder (air dry)	<i>Amaranthus gangeticus</i> .. ..	11.20	29.05



TABLE I—concl'd.

Name of foodstuff.	Botanical name.	Moisture per cent.	'Crude protein' per cent.
Amaranth leaves powder (fresh) ..	<i>Amaranthus gangeticus</i> ..	(85·80)	(4·90)
Ipomœa leaves powder (air dry)	<i>Ipomœa reptans</i> ..	10·82	29·33
„ „ (fresh) ..	„ ..	(90·40)	(2·90)
Lady's fingers powder (air dry)	<i>Hibiscus esculentus</i> ..	10·51	14·94
„ „ (fresh) ..	„ ..	(88·00)	(2·20)
Brinjal powder (dry) ..	<i>Solanum melongena</i> ..	10·94	14·03
„ „ (fresh) ..	„ ..	(91·50)	(1·31)

## TECHNIQUE AND FORMULÆ USED.

The technique employed is similar to that used by Chick *et al.* (1935*a* and *b*). Martin and Robison (1922), Mitchell (1924*b*), and Chick *et al.* (*loc. cit.*) have fully discussed the various problems involved in the calculation of the biological value and digestibility co-efficient, from the data obtained by the nitrogen-balance method.

The formulæ used by Chick and her co-workers, which have been adopted here, are as follows:—

I. The relative 'biological value' (B.V.) of a protein (p) is expressed as:—

$$100 \times \frac{\text{Body N saved}}{\text{Food N absorbed}} \quad \text{i.e., } 100 \left[ \frac{U_p - U_e}{I_p - (F_p - F_e)} \right]$$

where  $I_p$ ,  $U_p$ , and  $F_p$  are the daily N intake, urinary N excretion, and faecal N excretion respectively on a diet containing protein, and  $U_e$  and  $F_e$  are the daily endogenous N excreted in urine and faeces respectively on a nitrogen-free diet;  $F_p - F_e$  represents the nitrogen in the faeces derived from undigested food protein and  $I_p - (F_p - F_e)$  the true N intake on a diet containing protein.

II. The relative 'digestibility co-efficient' (D.C.) of a given protein (p) is expressed as:—

$$100 \left[ \frac{\text{Food N digested}}{\text{Food N intake}} \right] \quad \text{i.e., } 100 \left[ 1 - \frac{(F_p - F_e)}{I_p} \right]$$

## EXPERIMENTAL.

A group of 5 male rats (weighing about 150 g. at the commencement of the experiment) was used. The weights of the rats increased during the period of experiment as is shown in the figures given in Table III. It does not seem likely that the relatively small differences in weight of the rats in the various experiments would materially affect the comparability of the metabolism data obtained. The experimental diets containing the test materials and the nitrogen-free diet are shown in Table II:—

TABLE II.

*Composition of the diets used.*

	Nitrogen free g.	Milled raw rice g.	Whole wheat g.	Cambu g.	Cholam g.	Ragi g.	Lentil g.	Green gram g.	Soya bean g.	Drum- stick leaves g.	Amar- anth leaves g.	Ipomoea leaves g.	Lady's fingers g.	Brinjal g.
Foodstuff tested	..	75.0	42.0	50.0	48.0	70.0	42.0	44.0	25.0	19.0	17.0	17.0	33.3	35.6
Starch ..	71.2	..	32.0	26.0	26.0	8.0	33.0	31.0	50.0	53.0	53.0	55.0	40.0	38.0
Sugar ..	8.0	13.0	10.0	10.0	12.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0
Beef dripping ..	13.0	13.0	13.0	13.0	13.0	13.0	13.0	13.0	10.0	13.0	13.0	13.0	13.0	13.0
Salt mixture ..	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0
Cod-liver oil ..	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0
Calcium carbonate	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8
Percentage of N (dry weight).	0.0092	0.8192	0.8480	0.8560	0.8730	0.8624	1.7280	1.7120	1.0768	0.8800	0.8400	0.8403	0.8630	0.8736
* Crude protein* N x 6.25 dry weight.	..	5.12	5.30	5.35	5.46	5.39	10.80	10.70	10.48	5.50	5.25	5.25	5.39	5.46

Before the first test diet was given the rats received the nitrogen-free diet for 7 days; feeding with this diet was repeated at various intervals, usually after three-weekly test periods. The test diets were fed for a period of 7 days, in the order indicated in Table III. The first 3 days of each period is regarded as preparatory, allowing the rats to accommodate themselves to the new diets; the collection of urine and faeces was confined to the remaining 4 days of each period. The rats were given 'rest' for 4 days after each experimental period with the nitrogen-free diet as well as with the test diets. During the 'rest' period they received the stock diet. The level of protein intake was kept at approximately 5 per cent in the diets containing the cereals and vegetables and at 10 per cent in those containing pulses, since the protein content of the former is relatively lower. Results tend to be higher at a low level of protein intake. The figures obtained will, however, serve for drawing comparisons among the cereals and vegetables themselves.

The experimental diets were prepared by adding the necessary ingredients in the desired proportions, as shown in Table II. In addition, 3 c.c. of an aqueous solution of yeast extract (corresponding to 0.75 g. of dry yeast) containing the vitamin-B complex were given daily to each rat along with food. The diets were thoroughly mixed up, made into a paste by adding distilled water in order to prevent scattering of food, and samples were analysed for their moisture and protein content. The dry weight of the diets before the addition of water was noted and also the wet weight in the pasty condition. In weighing out the daily food, care was taken that the food given should not greatly exceed the average food consumption, thus avoiding a large residue. Food residue remaining uneaten was carefully collected daily, completely dried in an air oven at 100°C., and corresponding weights of the residual food subtracted from the dry weight of the food given daily to the rats. By this means daily food intake was obtained.

The urine and faeces were collected daily. To prevent decomposition of urine and consequent loss of ammonia, 20 c.c. of 5 per cent dilute sulphuric acid containing 2 per cent of carbolic acid and 1 per cent of thymol were placed in the flasks used to collect urine. The metabolism cages, funnels, separators, and flasks placed for collection of urine were washed down daily with small quantities of distilled water and the washings were added to the urine bottle. At the end of each experiment the urine and washings were filtered through muslin into a 500 c.c. standard flask, the volume made up to mark and then transferred into a clean stoppered bottle. Aliquot portions were removed for nitrogen estimation. The faeces were transferred daily into a small wide-mouthed bottle provided with a lid. At the end of each experiment the faeces were moistened with 10 c.c. of 5 per cent oxalic acid, dried in an air oven at 100°C. for a number of hours, cooled and then powdered in a glass mortar. The powdered faeces were then transferred to a small wide-mouthed weighed bottle, dried again at 100°C. and the weight again determined. The difference between the two weights gave the weight of the faeces. Aliquot portions were weighed out for nitrogen estimation. All the nitrogen estimations were done by the Kjeldahl method. In the calculation of the biological values and digestibility co-efficients, the average of the figures obtained for the endogenous urinary and faecal nitrogen at the beginning and end of a group of experiments was taken for that group, since no regular variations were observed in their value.

The data regarding the metabolism experiments are given in Table III:—









TABLE III—*contd.*

(Figures of intake and excretion represent daily averages.)

Rat number.	Initial body-weight g.	Final body-weight g.	Food intake g.	N intake mg.	Urinary N mg.	Faecal N mg.	Body N saved mg.	Food N absorbed mg.	Biological value per cent.	Digestibility co-efficient per cent.
<i>Period 9.—Green-gram diet N = (1.7120) per cent.</i>										
1	184	183	13.13	224.8	125.4	48.0	108.4	196.3	55	55
2	214	217	15.52	265.7	159.4	54.1	116.5	232.0	50	87
3	182	193	14.11	241.5	140.0	56.8	96.8	201.3	48	83
4	193	191	12.81	219.3	135.2	46.3	91.5	188.0	49	86
5	174	175	11.33	194.0	112.8	43.1	90.0	166.8	54	86
								Average ..	51	86
<i>Period 10.—Soya-bean diet N = (1.6768) per cent.</i>										
1	192	195	13.33	223.9	110.4	73.8	96.7	169.6	57	76
2	228	222	12.65	212.5	118.8	49.1	108.9	183.8	59	85
3	198	196	10.98	184.5	100.5	69.0	67.2	132.1	51	72
4	179	179	11.18	187.8	103.9	58.4	79.2	144.4	55	77
5	181	180	9.73	163.5	94.8	62.0	58.6	117.4	50	72
								Average ..	54	76







TABLE III—*contd.*

(Figures of intake and excretion represent daily averages.)

Rat number.	Initial body-weight g.	Final body-weight g.	Food intake g.	N intake mg.	Urinary N mg.	Faecal N mg.	Body N saved mg.	Food N absorbed mg.	Biological value per cent.	Digestibility co-efficient per cent.
<i>Period 15.—Nitrogen-free diet N = (0.0092) per cent.</i>										
1	222	215	14.10	..	42.0	22.7	..	..	..	..
2	240	237	15.20	..	47.8	25.8	..	..	..	..
3	180	179	12.95	..	31.9	19.4	..	..	..	..
4	190	185	12.78	..	44.1	16.8	..	..	..	..
5	192	184	12.43	..	41.3	18.4	..	..	..	..

*Period 16.—Lady's fingers diet N = (0.8630) per cent.*

1	223	213	16.03	138.3	58.8	57.3	87.1	103.3	84	75
2	246	238	16.73	144.5	67.4	63.2	85.7	106.0	81	73
3	186	187	11.93	103.1	50.6	51.8	55.4	71.3	78	69
4	202	193	11.23	97.0	52.2	50.1	53.9	63.9	84	67
5	195	188	15.10	130.5	56.7	59.3	74.5	89.2	84	68
Average ..									82	70

TABLE III—*concl.**(Figures of intake and excretion represent daily averages.)*

Rat number.	Initial body-weight g.	Final body-weight g.	Food intake g.	N intake mg.	Urinary N mg.	Faecal N mg.	Body N saved mg.	Food N absorbed mg.	Biological value per cent.	Digestibility co-efficient per cent.
<i>Period 17.—Brinjal diet N = (0.8736) per cent.</i>										
1	221	218	13.43	116.8	70.4	49.9	61.4	89.2	67	76
2	246	235	14.30	124.4	71.6	52.5	72.1	96.6	75	78
3	189	181	10.20	88.7	55.3	40.3	47.8	68.4	70	77
4	195	186	11.45	99.6	61.3	45.5	52.0	71.1	73	71
5	191	187	12.03	104.7	64.4	47.2	53.1	75.5	70	72
Average ..									71	75

*Period 18.—Nitrogen-free diet N = (0.0092) per cent.*

1	224	213	14.80	..	43.2	21.8	..	..	..	..
2	244	231	15.50	..	46.4	23.6	..	..	..	..
3	191	186	12.85	..	37.5	20.5	..	..	..	..
4	193	187	12.55	..	40.2	17.2	..	..	..	..
5	197	191	14.20	..	42.7	17.2	..	..	..	..

## DISCUSSION.

All the results reported in this paper relate to the total nitrogenous constituents of the foodstuffs examined, referred to as their 'crude protein' content. Many foodstuffs, e.g., vegetables, contain considerable amounts of non-protein nitrogen, part of which may exist as free amino acids, though in vegetables most of this is in the form of amides or amino acids, like asparagine, etc. The biological value of the proteins in a food may in most cases be enhanced by the supplementary effect of this non-protein nitrogen, consisting largely of the simpler, easily digestible, and assimilable peptides and amino acids and their derivatives (Grindley and Eckstein, 1916; Mitchell, 1924*a*; Bhagvat and Sreenivasaya, 1935).

*Cereals*.—There is no doubt that proteins of animal origin are as a class superior in biological value to those derived from plants. Cereal proteins, though inferior to animal proteins, nevertheless possess higher biological values than those of legumes. The proteins of rice are found to be distinctly superior in biological value to those of wheat, when the proteins are fed at the same level of intake. Wheat, however, contains nearly twice as much protein as rice. The biological values obtained, 80 for rice and 66 for wheat, agree well with the values reported by previous workers. Mitchell *et al.* (*loc. cit.*) reported a value of 86 for rice and 67 for wheat, while Boas-Fixsen *et al.* (*loc. cit.*) obtained a value of 68 for wheat. The proteins of cambu, cholam, and ragi possess higher biological values than those of wheat, but their digestibility co-efficients are lower. The proteins of cambu and cholam are of almost equal biological value (83) but the proteins of ragi, having a biological value of 89, appear to be the best of all cereal proteins for the maintenance of nitrogenous equilibrium in adult rats. The biological value (89) obtained for ragi proteins is in close agreement with the value (90.5) reported by Niyogi *et al.* (1934). On the basis of the present experiments, the cereal proteins range themselves as regards biological value in the following descending order: ragi, cholam, cambu, rice, and wheat.

*Legumes*.—Legumes in general contain a high percentage of protein and so form an important source of proteins in Indian diets; the proteins of legumes, however, were found to be the poorest in value of all those investigated, which is probably due to their deficiency in the essential amino acid cystine (Jones *et al.*, 1922, 1924). It was pointed out in the introduction that the biological values obtained by the previous workers for lentil and green gram are conflicting, Niyogi *et al.* (1934) reporting values of 58 and 60 and Basu *et al.* (*loc. cit.*) 32 and 52, respectively. The values obtained in the present investigation, 41 for the proteins of lentil and 51 for the proteins of green gram, agree more closely with those reported by Basu *et al.* The proteins of soya bean were found to be superior to those of green gram and lentil, and in this respect soya bean appears to resemble the nuts and oil-seeds, such as pea-nuts and coco-nuts, the biological values of whose proteins have been assessed at 58 and 58 respectively by Mitchell *et al.* (*loc. cit.*). The biological value (54.4) obtained for the proteins of soya bean in the present investigation is lower than that (64) reported by Mitchell *et al.* (*loc. cit.*). From the present experiments the pulse proteins tested may be arranged in the following descending order of merit as regards their biological value: soya bean, green gram, and lentil.

*Vegetables*.—Though vegetables do not contribute largely to the total proteins of the diet, they are in proportion to their calorie-yield surprisingly rich in protein.

On an air-dry basis, vegetables contain much more proteins than cereals and some of them contain as much protein as the pulses, e.g., amaranth leaves 29.05, ipomœa leaves 29.33, drumstick leaves 26.85, lady's fingers 14.94, and brinjal 14.03. Our information regarding the quality of the proteins in vegetables is scanty. Sherman (1933) remarks: 'That the dry legumes are both absolutely and relatively rich in protein is a fact so well recognized as not to require elaboration. Less generally realized is the fact that while the green vegetables contain too much water to show high absolute values or percentages by weight of protein, yet they show as much or more of the total fuel value in the form of protein as is customary or desirable in ordinary dietaries. The vegetables differ among themselves in the nutritive efficiency of their proteins. Probably the proteins of the leafy vegetables are, weight for weight, of somewhat higher nutritive value than the proteins of seeds. Even among the latter, there are also differences, which naturally have been studied mainly in the legumes, since they are so generally regarded as significant largely for their protein content'.

The substantial truth of these remarks is confirmed by the results of the present investigation and by the work of Adolph *et al.* (*loc. cit.*) on cabbage and sweet potato proteins. Of the five vegetables investigated, four possess proteins of high biological value: lady's fingers 82, amaranth 72, brinjal 71, and ipomœa leaves 67, which are thus superior in nutritive value to the proteins of legumes. The proteins of drumstick leaves, like those of lentil, are of low biological value (41). In experiments in which the level of protein intake was 10 per cent, Adolph *et al.* (*loc. cit.*) observed a biological value of 76 for the crude isolated proteins of cabbage leaves and a value of 72 for the crude isolated proteins of sweet potato. Mitchell *et al.* (*loc. cit.*) recorded a biological value of 67 for the total proteins of potato, which were fed at an 8 per cent level, while Kon (1928) obtained a value of 71 for 'tuberin', the chief protein of potato. From the above results it may be concluded that the proteins of most of the vegetables so far investigated are of high biological value and probably are of value in supplementing the proteins of cereals and pulses. The vegetable proteins investigated fall into the following descending order of merit as regards their biological value: lady's fingers, amaranth leaves, brinjal, ipomœa leaves, and drumstick leaves.

TABLE IV.

*The available or net protein content of certain foodstuffs.*

Name of foodstuff.			Moisture per cent.	'Crude protein' per cent.	Level of protein intake per cent.	Biological value per cent.	Digestibility co-efficient per cent.	Available or net protein per cent.
Raw polished rice	..		12.50	6.88	5	80	97	5.34
Wheat	..	..	12.31	12.62	5	66	93	7.75
Cambu	..	..	12.10	10.48	5	83	89	7.74
Cholam	..	..	11.20	10.27	5	83	91	7.76

TABLE IV—concl'd.

Name of foodstuff.	Moisture per cent.	'Crude protein' per cent.	Level of protein intake per cent.	Biological value per cent.	Digestibility co-efficient per cent.	Available or net protein per cent.
Ragi .. ..	12.58	7.12	5	89	80	5.07
Lentil .. ..	12.44	25.7	10	41	88	9.27
Green gram .. .	10.42	23.8	10	51	86	10.44
Soya bean .. ..	9.30	40.0	10	54	76	16.42
Drumstick leaves (dry) ..	10.68	26.85	5	41	77	8.48
„ „ (fresh) .	(75.00)	(6.65)	..	..	..	(2.72)
Amaranth leaves (dry) ..	11.20	29.05	5	72	78	16.31
„ „ (fresh) ..	(85.80)	(4.90)	..	..	..	(2.75)
Ipomœa leaves (dry) ..	10.82	29.33	5	67	85	16.70
„ „ (fresh) ..	(90.40)	(2.90)	..	..	..	(1.65)
Lady's fingers (dry) ..	10.51	14.94	5	82	70	8.58
„ (fresh) ..	(88.0)	(2.2)	..	..	..	(1.26)
Brinjal (dry) .. ..	10.94	14.03	5	71	75	7.47
„ (fresh) .. ..	(91.50)	(1.31)	..	..	..	(0.70)

## THE 'AVAILABLE OR NET PROTEIN CONTENT' OF FOODSTUFFS.

The term 'available or net protein content' of a given foodstuff denotes the actual amount of protein utilized by the body for every 100 g. of foodstuff intake. For a complete determination of this figure, it is necessary to consider (1) the total nitrogen content of the foodstuff, i.e., the crude protein content ( $N \times 6.25$ ), (2) the loss or wastage of protein nitrogen in metabolism, i.e., during the process of its conversion into tissue constituents or body secretions, and (3) the loss or wastage of protein nitrogen in digestion. 'The available or net protein content' of a foodstuff, therefore, depends as much upon its 'crude protein' content as upon the extent of its utilization in digestion and in anabolism, as shown by Mitchell and Beadles (1927). It may be calculated by the combined use of the above three factors, viz., the 'crude protein' content, the digestibility co-efficient, and the biological value. Mitchell's formula is as follows:—

'Available or net protein content' =

$$\text{'crude protein' per cent} \times \frac{\text{Digestibility co-efficient}}{100} \times \frac{\text{Biological value}}{100}$$

The 'available protein content' of the 13 foodstuffs investigated is given in Table IV. Just for comparison, some data regarding the protein content, digestibility co-efficient, biological value, and 'available protein' or 'protein value' of some other foodstuffs are reproduced in Table V from Mitchell and Hamilton's book 'The Biochemistry of Amino Acids', p. 556.

TABLE V.

*'Protein value' of foods form maintenance and growth on an 8 to 10 per cent level of protein intake (Mitchell and Hamilton, 1929).*

Food.	Moisture.	PROTEIN CONTENT PER CENT.		Digestibility co-efficient per cent.	Biological value per cent.	AVAILABLE PROTEIN CONTENT PER CENT.	
		Fresh basis.	Dry basis.			Fresh basis.	Dry basis.
Whole egg ..	73.2	13.2	49.3	100	94	12.0	44.9
Milk ..	87.0	3.3	25.4	100	85	2.6	20.2
Beef liver ..	71.2	20.4	70.8	90	77	14.9	51.1
Rolled oats ..	7.7	16.7	18.1	90	65	9.8	10.6
Whole wheat ..	11.4	13.8	15.6	91	67	7.1	8.1
Whole corn ..	10.3	7.5	8.4	95	60	3.0	3.5
Potato ..	78.3	2.2	10.1	78	67	0.8	3.9
Navy beans ..	12.6	22.5	25.7	76	38	4.2	6.0
Cocoa ..	4.6	21.6	22.6	38	37	1.6	1.8

If results obtained with rats are applicable to human beings, the 'available or net protein' content of a foodstuff is of importance in planning diets. As a rule, the protein content of diets is calculated by consulting tables of chemical analysis of foodstuffs, which give 'crude protein' content. The possible error involved in such calculations may be illustrated as follows: lentil, which contains 25.7 per cent of 'crude protein' of which 88 per cent only is digestible, contains 22.4 per cent of digestible protein. Since only 41 per cent of the digestible protein can be used to cover the protein requirements of the body at a 10 per cent level of protein intake, lentil contains only 9.27 per cent of 'available or net protein'. Suppose a diet is planned which according to tables based on chemical analysis contains 25.7 g. of protein derived from lentil alone. Only 9.27 g. of that protein are available for utilization by the body and the remaining 16.43 g. of protein are wasted in digestion and metabolism. Green gram contains 23.8 per cent of 'crude protein' of which 86 per cent is digestible; of the latter only 51 per cent is available for body purposes, so that this food contains only 10.23 per cent of 'available protein'. Navy beans (Mitchell and Hamilton, *loc. cit.*), with 22.5 per cent of 'crude protein', contain only



4.2 per cent of 'available protein'. It is obvious, from the above examples, that in practical dietetics the quality of the proteins should be taken into consideration, as well as the quantity, since all proteins, especially proteins of vegetable origin, are not of the same quality.

It must be admitted that in basing the protein value of a foodstuff on its available or net protein content, no consideration is given to the supplementary relationships which may exist between different proteins in a diet and to the fact that different proteins may have different supplementary values.

### SUMMARY.

1. The biological values and digestibility of the proteins of 13 foodstuffs, which include 5 cereals—rice, wheat, cambu, cholam, and ragi; 3 pulses—lentil, green gram, and soya bean; and 5 vegetables—drumstick leaves, amaranth leaves, ipomœa leaves, lady's fingers, and brinjal, have been determined by the balance-sheet method on adult male rats.

2. On a 5 per cent level of protein intake, the digestibility co-efficient and biological value of rice are 97 and 80, of wheat 93 and 66, of cambu 89 and 83, of cholam 91 and 83, and of ragi 80 and 89, respectively. By taking into consideration both the digestibility co-efficient and biological value, the cereal proteins may be arranged in the following descending order of merit: rice, cholam, cambu, ragi, and wheat.

3. At a 10 per cent level of protein intake the digestibility co-efficient and biological value of lentil are 88 and 41, of green gram 86 and 51, and of soya bean 76 and 54, respectively. Taking into consideration both the digestibility co-efficient and biological value, the pulse proteins may be arranged in the following descending order of merit: green gram, soya bean, and lentil.

4. At a 5 per cent level of protein intake, the digestibility co-efficient and biological value of the proteins of drumstick leaves are 77 and 41, of amaranth leaves 78 and 72, of ipomœa leaves 85 and 67, of lady's fingers 70 and 82, and of brinjals 75 and 71, respectively. On a similar basis the proteins of vegetables may be arranged in the following descending order of merit: lady's fingers, ipomœa leaves, amaranth leaves, brinjal, and drumstick leaves.

5. As regards 'available or net protein content', the cereals range themselves in the following descending order: cholam, wheat, cambu, rice, and ragi; the pulses: soya bean, green gram, and lentil, and the vegetables (in a fresh state): amaranth leaves, drumstick leaves, ipomœa leaves, lady's fingers, and brinjal.

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## BALANCED DIETS.\*

### Part I.

BY

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THE fact that the low standard of health among the bulk of the Indian population is not so much due to under-feeding as to ill-feeding has been widely acknowledged only recently. The problem of finding a cheap and complete dietary, paying due heed, of course, to the local conditions and habits, has been engaging the attention of scientific as well as social workers. The Bombay Presidency Baby and Health Week Association (1935) have been trying to disseminate some useful information concerning the value of foodstuffs in common use in this part of the country. The Association has also formulated from time to time certain diets to meet the requirements of an individual at a maximum monthly cost of Rs. 6 per adult at the market rate of the food constituents. These diets are called the 'balanced diets' and provision is made in these for the fat, protein, carbohydrate, and caloric requirements of a moderately hard-working individual. Diets at an average monthly cost of Rs. 7 are also included for those who can afford to spend more.

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\* The subject-matter of this paper forms a part of the investigations on the nutritive value of certain diets, which are carried out by a Committee under the joint auspices of the Bombay Presidency Baby and Health Week Association and the Seth Gordhandas Sunderdas Medical College, Bombay, consisting of Doctors H. V. Tilak, B.Sc., M.B., B.S., F.R.C.S.E., and K. S. Mhaskar, M.D., D.P.H., representing the Association, and of Doctors Jivraj N. Mehta, M.D. (Lond.), M.R.C.P. (Lond.), S. P. Niyogi, M.Sc., M.B. (Cal.), and V. N. Patwardhan, M.Sc., PH.D. (Lond.), representing the College. The Committee is also assisted by Dr. A. K. Boman-Behram, M.B., B.S., and Mr. R. G. Chitre, M.Sc. The Indian Research Fund Association and the Sir Dorabji Tata Memorial Trust have helped the investigation by substantial grants.

Some experiments conducted by the Association on the boys and girls at the Byramjee Jeejeebhoy Home, Matunga, Bombay, showed that those fed on the 'balanced diet' grew better than the others who were on the routine diet of the home. It was with a view to investigating scientifically the adequacy of this diet and improving upon it wherever necessary that the present investigation was undertaken. A complete study of the vegetarian and non-vegetarian diets would be necessary; the present paper represents the laboratory experiments on non-vegetarian diets only. The authorities of the David Sassoon Industrial School for boys, Matunga, Bombay (hereafter referred to as D. S. I. School), have extended their co-operation, making it possible to conduct controlled experiments on the inmates.

For purposes of comparison another diet has been evolved in the laboratory by taking into account the commonly accepted (Report of the Committee on Nutrition appointed by the British Medical Association, 1933) human requirements of fats, proteins, carbohydrates, calories, vitamins, and minerals. In the case of fats and proteins more attention has been paid to the inclusion in the diet of substances of animal origin in sufficient amounts. This diet has been named the 'physiologically ideal diet', its calculated cost at market rates amounts to Rs. 10 per month per adult. The 'routine diet' of the D. S. I. School, the cost of which at market rates is Rs. 4-8 per month per adult, served as control. In the following pages these diets will be referred to as P. I. D., B. D. and R. D.

Feeding experiments on the inmates of the D. S. I. School and the growth tests on albino rats were carried out simultaneously with the non-vegetarian diet. This communication deals with the animal experiments. The experiments on boys which are being conducted by Dr. K. S. Mhaskar and Dr. A. K. Boman-Behram will form the subject of a later communication.

Attempts to find cheap and well-balanced diets are progressing along somewhat similar lines in other parts of India. Wilson, Ahmad and Mullick (1936) have made a diet survey of some middle-class families and some institutions in Calcutta; they found that the cost of an adequate diet, per man value, per day, at Calcutta was between 4.4 and 5.6 annas entailing a monthly expenditure of Rs. 7 to Rs. 10 per head. Aykroyd and Krishnan (1936) have examined at Coonoor a number of cheap diets. They have investigated the effect of including in the diet the staple cereals like rice, wheat, jowar (*Sorghum vulgare*), bajri (*Pennisetum typhoideum*), and ragi (*Eleusine coracana*) in various proportions supplemented by legumes, root and leafy vegetables, dried skim-milk, butter-milk, and jaggery. From among several cheap diets examined the one on which they obtained the best growth was constituted as follows:—

			Oz.
Ragi ( <i>Eleusine coracana</i> )	..	..	16
Soya bean	..	..	2
Dhal arhar ( <i>Cajanus indicus</i> )	..	..	2
Jaggery	..	..	1
Leafy vegetables	..	..	8
Root vegetables	..	..	2
Coco-nut oil	..	..	1.5
Butter-milk	..	..	6

The average weekly increase in weight of 12 young rats during 10 weeks on this diet was 9·8 g., the corresponding average for their stock diet being 10·6 g.

## EXPERIMENTAL.

*The diets.*—In Table I is shown the composition of the diets per adult person under investigation. A few changes were made in the 'balanced diet' during the course of the investigation; the composition of both of these is given in the table:—

TABLE I.

Article.	QUANTITY IN OUNCES.			
	P. I. D.	B. D.		R. D.
		Original.	Modified.	
Polished rice .. ..	6	10	9	8
Whole-wheat flour .. ..	6	6	8·5	7
Jowar ( <i>S. vulgare</i> ) .. ..	4	..	..	..
Bajri ( <i>P. typhoideum</i> ) .. ..	..	..	..	5
Pulses .. ..	1	1	1·7	4
Soya bean .. ..	..	1·5	1·5	1
Rice bran .. ..	..	1·0	1·0	..
Ground-nut oil cake .. ..	..	0·5	0·5	..
Vegetable oil .. ..	1	1	1	1
Mutton fat .. ..	1	1	1·3	..
Ghee .. ..	0·5	..	..	..
Dried skim-milk .. ..	2	0·5	1·5	..
Fresh whole milk .. ..	6	..	..	..
Eggs .. ..	1	..	..	..
Meat and fish .. ..	3	3	3	0·5
Sugar and jaggery .. ..	2	1·7	1·5	2
Root vegetables .. ..	8	5·5	3	10
Leafy vegetables .. ..	6	3·5	5·5	..
Lemons and tomatoes .. ..	4	..	..	..

*The collection and analyses of the diets.*—The diets were cooked in the Industrial School and brought over to the laboratory where they were dried at 40°C. to 45°C. in a current of hot air for about eight to ten hours. The material was then powdered and kept stoppered at room temperature. The diets thus collected were analysed from time to time to keep a check on the distribution and also to make certain that the analytical figures agreed with those calculated from the data given by McCarrison (1931). Table II shows the results of the actual analyses:—

TABLE II.

*Analyses of the experimental diets showing the total quantities ingested per day per adult.*

Constituent.	P. I. D.	B. D.		R. D.
		Original.	Modified.	
	g.	g.	g.	g.
Proteins, total .. ..	103.2	87.6	100.5	88.7
" of animal origin .. ..	(51.6)	(29.7)	(36.9)	(4.2)
Fats, total .. ..	82.5	71.5	81.6	31.7
" of animal origin .. ..	(52.5)	(30.8)	(40.2)	(0.4)
Carbohydrates .. ..	464	472	446	586
Crude fibre .. ..	11.0	12.1	14.3	14.9
Iron (Fe) .. ..	0.19	0.16	0.13	0.23
Calcium .. ..	2.57	1.43	1.74	2.55
Magnesium .. ..	1.11	1.16	1.08	1.2
Phosphoric acid ( $H_3PO_4$ ) .. ..	6.57	6.33	6.66	5.1
Sulphuric acid ( $H_2SO_4$ ) .. ..	2.89	1.77	1.53	1.65
Chlorides as NaCl .. ..	14.16	15.0	14.43	14.3
Insoluble matter .. ..	0.76	0.50	0.49	0.97
Total calorific value of the diet	3,000	2,960	3,000	3,060

The capacity of a protein or a mixture of proteins to promote growth and maintain an animal in health will be determined by its content of those amino acids which are indispensable for growth and maintenance. Evidence reviewed by Mitchell and Hamilton (1929) shows that lysine, cystine, and tryptophane can be considered as essential amino acids. The indispensability of tyrosine has not been proved beyond doubt. Arginine and histidine seem to be interconvertible in the animal body. The amounts of all the six, however, have been determined.

The essential amino acids were estimated after hydrolysis of the fat-free diet with hydrochloric acid (1:1). The mono-amino acids were separated from the di-amino acids by the precipitation of the latter with phosphotungstic acid by Hausmann's method as modified by Osborne and Harris (1903). The amounts of cystine, lysine, histidine, and arginine were determined according to van Slyke's (1915) method of nitrogen distribution in the phosphotungstic acid precipitate.

Tyrosine was determined in an aliquot portion of the original hydrolysate by the method of Folin and Denis (1912). Tryptophane was calculated on the basis of humin nitrogen obtained on acid hydrolysis (Gortner and Holmes, 1917).

TABLE III.

*Essential amino acid content of the diets.*

Amino acid.	P. I. D. per cent.	B. D. (original) per cent.	R. D. per cent.
Tyrosine ..	0.13	0.10	0.11
Arginine ..	0.80	0.72	0.72
Lysine ..	0.20	0.05	0.07
Histidine ..	0.68	0.87	0.78
Cystine ..	0.59	0.49	0.51
Tryptophane ..	0.51	0.64	1.10

*Influence on the growth of rats.*—Young growing rats from healthy litters four to five weeks old were selected for this experiment. Fifteen rats were kept on the P. I. D., fourteen on B. D., and eleven on R. D. To eliminate errors due to any

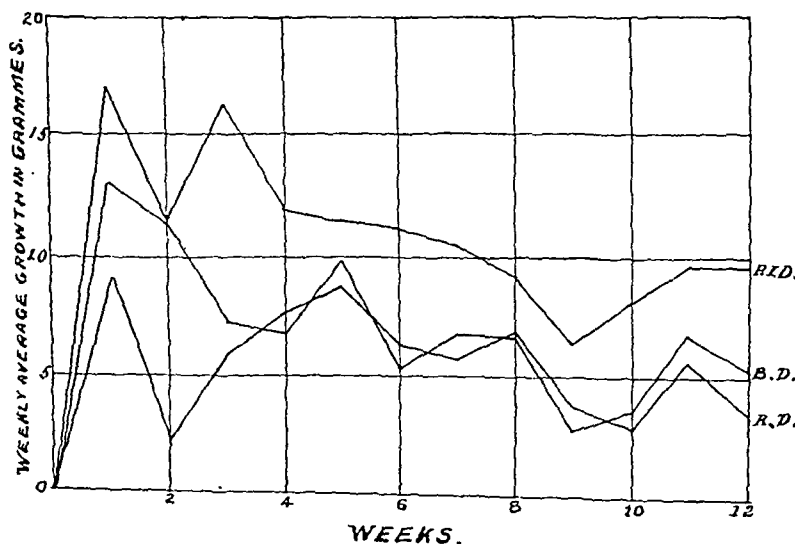


Fig. 1. Showing the average weekly increase in weights of rats on experimental diets.

similarity of response among litter mates the number in each group was made up by taking rats from different litters. Males and females were segregated. Food and water were given *ad lib*. Rats were weighed twice every week for a period of 12 weeks. In Fig. 1 is plotted the average weekly increase in weight in each group.

In Fig. 2 are shown the best growths obtained in a male and a female rat from each group.

At the end of March 1936 some alterations were made in the constituents of the B. D.; the modified diet is shown in Table I. The main alteration consisted of the addition of an extra ounce of skim-milk powder which required minor adjustments in other directions. Slight alterations were also made in the P. I. D. to make good the loss which occurred during the process of cooking. These alterations,

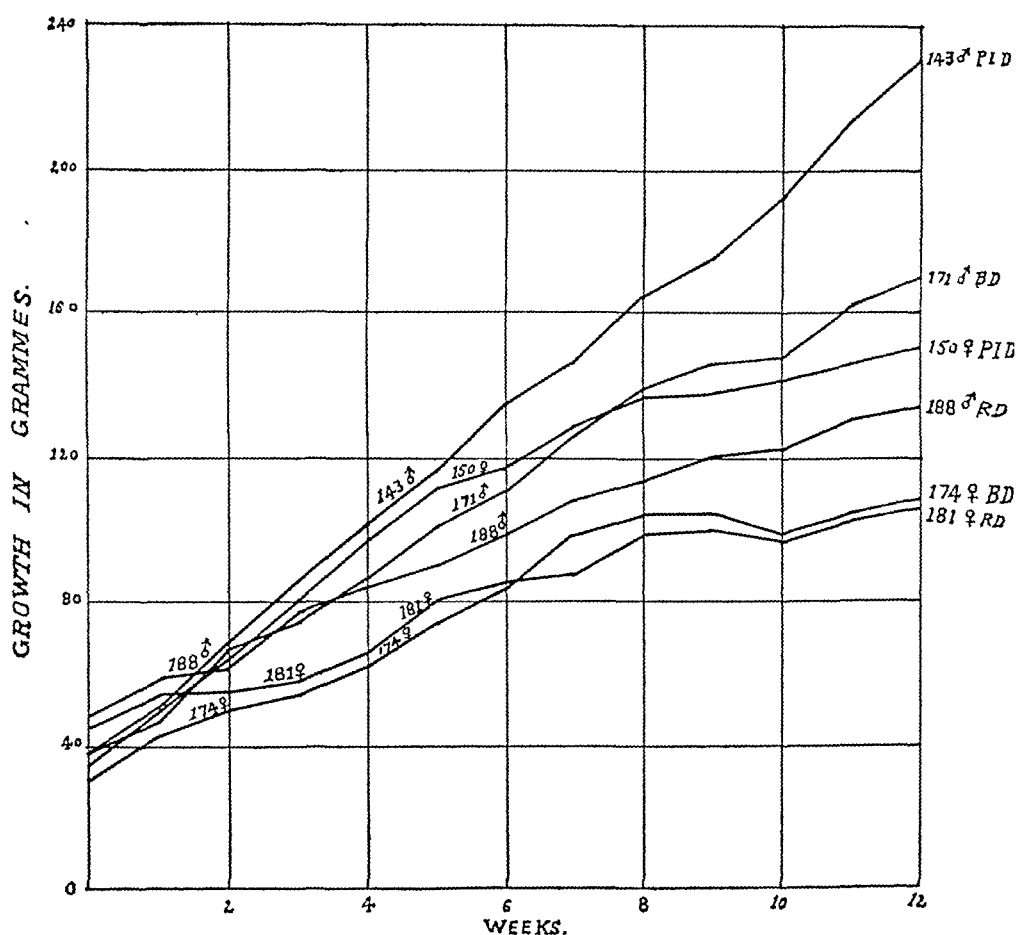


Fig. 2. Showing the maximum growth of a male and a female from each diet group.

however, did not materially affect the caloric value of the diet. Feeding experiments with B. D. on albino rats were started in three batches in early February and early March of 1936 with the result that all the rats belonging to the B. D. group had been on this modified diet for periods ranging from four to eight weeks. Reference will be made to this fact later.

*Influence on reproduction.*—When the rats had attained the age of 120 to 130 days males and females were brought together, one pair in each cage. The number of days elapsing before the arrival of the litters and the number of young rats in



each litter were noted. The mothers were separated from the young rats after three weeks and mating repeated with the same male and similar observations repeated. The results are given in Table IV:—

TABLE IV.

*Influence of diet on the reproductive ability of rats.*

Diet.	Serial number of female rat.	NUMBER OF DAYS FROM MATING TO BIRTH OF THE LITTER.		NUMBER OF YOUNG RATS IN THE LITTER.	
		1st.	2nd.	1st.	2nd.
P. I. D. ..	146	25	24	7	11
.. ..	144	24	..	5	..
.. ..	148	26	24	9	4
.. ..	191	25	25	7	9
.. ..	192	24	28	6	10
B. D. ..	174	23	25	4	3
.. ..	183	39	..	6	..
.. ..	196	24	32	6	4
.. ..	198	48	27	6	9
.. ..	199	43	..	6	..
R. D. ..	181	69	..	6	..
.. ..	180	76	..	6	..
.. ..	182	98	..	3	..

## DISCUSSION.

From Fig. 1 which shows the average weekly increase in weight during 12 weeks, it is evident that the 'balanced diets', both original and modified, fall far below the standard for normal growth in rats as shown by that attained on the 'ideal diet'. They seem to be almost the same in their effect on growth as the 'routine diet' of the D. S. I. School. The averages for all 12 weeks for the three diets are as follows:—

Diet.	Average weekly increase in weight during 12 weeks in grammes.
1. Physiologically ideal diet (P. I. D.).	12.08
2. Balanced diet (B. D.)	7.13
3. Routine diet (R. D.)	5.70

Fig. 2 shows that the maximum response in growth to B. D. and R. D. is markedly lower than the response obtained with P. I. D. Here also the proximity of the growth curves on the former two diets is noticeable. Here reference must be made to the fact already mentioned that B. D. was modified early in April. The rats designed for feeding with B. D. were put on the experimental diet in three batches, on the 31st of January, 28th of February, and 13th of March, 1936. Thus, the first batch received the modified B. D. for the last four weeks, the second batch for the last six weeks and the third batch for the last eight weeks of the experimental period. Since the modifications were made to make the diets more nutritious, the change ought to have been reflected in the weekly increase in weights. That it is not so is amply shown by the graph for B. D. in Fig. 1.

The analytical figures in Table II show that the original B. D. and R. D. are poor in good quality fats and proteins, i.e., fats and proteins of animal origin. Inclusion of these in the diet in certain proportions is necessary as the former serve as a vehicle for the fat-soluble vitamins, while the latter effect economy of protein assimilation by providing the right type of proteins. An increase of animal protein from 29.7 to 36.9 per cent and of animal fat from 30.8 to 40.2 per cent in B. D. was not sufficient to produce any increase in the growth of rats. A quantitative assay of the essential amino acids in these diets (Table III) shows that with the exception of lysine, in which the original B. D. and R. D. are definitely poor, their distribution seems to be more or less even. Since information about the minimum intake of those amino acids required for growth or maintenance in human beings is not available, it is not possible to base any significant conclusion on the data in Table III beyond a mention that the lysine content might probably have been a limiting factor in determining the growth of rats.

During the whole of the experimental period the rats on P. I. D. showed no loss of hair and were generally healthy. Those on B. D. and R. D. lost a good deal of hair during the third and fourth weeks of feeding. Moreover, there were two deaths in the B. D. group, the causes of which could not be ascertained as no post-mortem examination was done.

It has not been possible to derive much information from the quantitative analyses of the diets for elements like iron, calcium, phosphorus, sulphur, and chlorine. All these seem to have been supplied in quantities above the minimum requirements postulated by Sherman (1923). From a scrutiny of Tables I, II, and III it appears that the better response to P. I. D. could possibly be ascribed to the additive influence of the following factors: (1) the high animal fat and protein content, (2) the inclusion of fresh whole milk, and (3) the high lysine content.

Since the 'balanced diet' is designed for consumption by adults it was thought desirable to follow up the growth of rats with observations on the effect of these three diets on their reproductive ability. Results given in Table IV show that the number of days which elapsed between the pairing of male and female rats and the arrival of the first litter varied according to the diet with which the rats were fed. The average number of such days for rats on P. I. D. was 24.8, for those on B. D. it was 36.4 and for the rats of the R. D. group the average was 81 days; the average number of young rats per litter in each group was 6.8, 5.6, and 5.0, respectively. The extreme slowness of response in case of the last diet may probably have been due to a variety of causes among which may be included (a) late maturity, (b) unsuccessful mating, or (c) resorption of the fertilized ovum. For want of further study no light can be thrown on this subject. Observations of this kind might, however, bring to light any effects which diets, planned or otherwise, may exert on the factors responsible for sexual reproduction.

#### SUMMARY.

The non-vegetarian 'balanced diets' advocated by the Bombay Presidency Baby and Health Week Association, a 'physiologically ideal diet' evolved in this Laboratory, and the 'routine diet' of the David Sassoon Industrial School for boys, Matunga, Bombay, have been examined analytically and also by observing their effect on the growth and reproduction of albino rats. On the basis of the experiments reported in the preceding pages, the following conclusions can be drawn:—

1. The 'balanced' and the 'routine' diets are relatively poor in fats and proteins of animal origin. The same diets are very poor in lysine which is an essential amino acid.
2. The average weekly increase in weights of rats on the 'balanced' and 'routine' diets was 7.13 g. and 5.7 g., respectively, as compared with 12.08 g. on the 'physiologically ideal' diet. A 10 per cent increase of animal fats and a 7 per cent increase of animal proteins in the original B. D. due to the addition of mutton fat and skim-milk powder, did not cause any rise in the average weekly increase in weights.

3. The reproductive power has been impaired only slightly in rats on the 'balanced' diet, but more markedly in rats on the 'routine' diet. Rats fed on the 'physiologically ideal' diet procreated according to expectations.

Further work on testing other cheap diets is in progress.

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## THE ASH, CALCIUM, AND PHOSPHORUS CONTENT OF SOME COMMON BENGALI FOODSTUFFS.

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THE significance of minerals in nutrition needs no stressing, and an opinion on the intake of those substances can only be made when an analysis of the common varieties of foodstuffs is available. Furthermore, modern investigation on dietary habits has shown that all over the world the phosphorus and above all the calcium intake is often below what can be absorbed by the healthy growing child. The figures for ash, calcium, and phosphorus given in this paper are merely representatives of some of the common varieties of foods eaten in Bengal and purchased in the Calcutta market. Apart from their use in practical dietetics in this province a comparison with similar foodstuffs in other areas in India may not be without value when taken in conjunction with the local dietaries and/or any signs of deficiency disease. The figures given in the Table on the whole need no particular comment except to emphasize one or two points which should be noted by those concerned in food planning on a moderate scale of means. Atta and the dals are in general richer in calcium and phosphorus than rice. The vegetables as a whole are not particularly good sources of calcium except perhaps cabbage and in a lesser degree bhindi and spinach. This, however, need not detract from their use as they contain other important minerals apart from the accessory food factors. Above all, however, milk and milk products such as curds, sandesh, etc., are by far the best sources of

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calcium and in addition as shown by Sherman (1932) calcium in the form of milk is much better absorbed than in a food such as spinach.

TABLE.

*Analysis of foods in g. per 100 g. material.*

Foodstuff.	Total ash (g.).	Total Ca (g.).	Total P (g.).
ANIMAL FOODS.—			
Mutton .. ..	0.96	0.019	0.242
Fish ( <i>hilsa</i> ) .. ..	0.96	0.102	0.352
„ ( <i>rôhita</i> ) .. ..	1.30	0.073	0.314
„ ( <i>tangra</i> ) .. ..	0.60	0.065	0.302
„ ( <i>kôî</i> ) .. ..	1.30	0.131	0.252
Egg, hen .. ..	0.96	0.045	0.180
MILK AND MILK PRODUCTS.—			
Milk, cow's .. ..	0.642	0.120	0.092
Curd .. ..	0.680	0.138	0.112
Sandesh .. ..	1.70	0.590	0.360
Butter .. ..	Trace	Trace	Trace
Ghee .. ..	„	„	„
CEREALS AND PULSES.—			
Wheat (atta) .. ..	1.60	0.097	0.310
Rice .. ..	0.75	0.040	0.083
Suji (semolina) .. ..	0.56	0.060	0.150
Gram, whole .. ..	2.70	0.190	0.320
„ dal .. ..	2.30	0.106	0.406
Arhar „ .. ..	3.20	0.059	0.476
Mung „ .. ..	3.60	0.141	0.592
Beans .. ..	0.75	0.060	0.110
Lentils (masur) .. ..	1.60	0.064	0.384

TABLE—*contd.*

Foodstuff.			Total ash (g.).	Total Ca (g.).	Total P (g.).
NUTS.—					
Almonds	..	..	2.16	0.072	0.401
Coco-nut	..	..	1.32	0.034	0.191
VEGETABLES.—					
Potato	..	..	0.93	0.007	0.060
Carrots	..	..	0.99	0.058	0.055
Radish	..	..	0.78	0.035	0.025
Cabbage	..	..	0.74	0.134	0.039
Spinach	..	..	1.50	0.070	0.080
Tomato	..	..	0.54	0.011	0.032
Cucumber	..	..	0.50	0.023	0.049
Pumpkin	..	..	0.23	0.029	0.020
Brinjal	..	..	0.42	0.028	0.035
Bhindi (lady's fingers)	..	..	0.96	0.100	0.105
Patòl	..	..	0.44	0.030	0.100
Karela (bitter gourd)	..	..	0.842	0.035	0.048
Vegetable marrow	..	..	0.41	0.020	0.010
Jhinga (tôri) (ridge gourd)	..	..	0.50	0.010	0.020

TABLE—concl'd.

Foodstuff.	Total ash (g.).	Total Ca (g.).	Total P (g.).
<i>VEGETABLES—concl'd.</i>			
Lau (gourd) .. ..	0.72	0.030	0.009
Puin sak .. ..	1.08	0.070	0.096
Onion .. ..	0.58	0.026	0.062
<i>FRUITS.—</i>			
Banana .. ..	0.68	0.043	0.041
Grapes .. ..	0.56	0.075	0.025
Oranges .. ..	0.32	0.068	0.026
Papaya .. ..	0.48	0.054	0.026
Mango pulp .. ..	0.65	0.021	0.019
Jack-fruit .. ..	0.71	0.027	0.065

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## THE CAROTENE CONTENT OF SOME COMMON BENGALI FOODSTUFFS.

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THE importance of carotene as a source of vitamin A in human nutrition in India should be appreciated by all those concerned in practical nutrition. Foods rich in preformed vitamin A, such as milk, fish-liver oils, eggs, etc., are in general not available for most of the population and indeed among the poorer classes, carotene in the form of vegetables is their only protection against deficiency disease associated with this vitamin. The necessity for figures giving the carotene content of local foods is hence obvious. The data recorded here were obtained from analyses of foods bought in the local Calcutta market and comprise the cheapest and most widely consumed products used by the people.

### METHOD OF CAROTENE ESTIMATION.

Five to ten grammes of material were ground up with pure sand and repeatedly extracted first with acetone and then with ether until no more of the pigments were removed and the extract was colourless. The combined extracts were transformed into a separating funnel, some more ether added and repeatedly washed with water to remove all the acetone. The ethereal solution was then saponified by shaking with 20 per cent alcoholic potash. It was then washed with water to remove the chlorophyll. If this solution still contained some green pigment, the saponification was repeated. The yellow ethereal extract containing only carotinoid pigments

was distilled under conditions preventing oxidation of the pigment (either by adding a trace of hydroquinone or in an atmosphere of an inert gas) until nearly the whole of the solvent was distilled off. The carotinoid pigments were then taken up in petroleum ether and repeatedly washed with 60 to 70 per cent alcohol to separate xanthophyll, etc. The petroleum ether extract was then washed once or twice with distilled water to remove any trace of alcohol, dried over anhydrous sodium sulphate, brought to a known volume and the carotene estimated colorimetrically. A Lovibond tintometer was used for the estimation. A standard curve of carotene units was first prepared from different known concentrations of pure carotene and all values were derived by reference to this curve.

In this paper (see Table) it is sufficient to point out foods which are rich in this constituent. Among three classes of foodstuffs, namely, vegetables, fruits, and cereals, only the former can be considered as effective sources of this material.

It will be seen that vegetables of the leafy type, particularly *saks*, are the richest; *soya sak*, for instance, contains some 10 mg. per 100 g. The tuberous vegetables on the other hand are relatively carotene-poor, except carrots. Radish leaves, for instance, contain about 8 mg., while the radish itself has almost no carotene. With the exception of the mango, the common fruits and cereals are for all practical purposes carotene-free.

As far as the practical application of the results is concerned, it should be pointed out that there is good reason to believe that carotene can replace the preformed vitamin in most respects, possibly all, but this is not yet an established fact. Further, the relative absorbability of carotene under different conditions and its transformation into the vitamin itself are both factors which must be taken into consideration in assessing its value. This, however, should in no way prevent its increased use, particularly as such substances as the *saks* are popular and easily grown in the plains or hills provided the minimum of moisture is present.

TABLE.

*The carotene content of some Bengali foods.*

Number.	Foodstuff.	Mg. carotene per 100 g. of material.
VEGETABLES.—		
1	Spinach .. ..	5.6-6.5
2	Pumpkin leaves ..	5.75-7.2
3	Polla sak .. ..	6.2-6.5
4	Soya sak .. ..	10.8-11.6
5	Lal sak .. ..	6.75
6	Meti sak .. ..	4.0-4.5
7	Puin sak .. ..	3.2-3.5

TABLE—*contd.*

Number.	Foodstuff.	Mg. carotene per 100 g. of material.
VEGETABLES— <i>contd.</i>		
8	Radish leaves .. ..	7.2-8.6
9	Lettuce .. ..	1.5-1.94
10	Mint ( <i>podina</i> ) .. ..	3.3-4.6
11	Cauliflower leaves .. ..	3.3-3.9
12	<i>Kalmi sak</i> ( <i>ipomoea</i> ) .. ..	5.2-5.5
13	Radish .. ..	Traces
14	Potato .. ..	"
15	Brinjal .. ..	"
16	Turnip .. ..	"
17	White turnip .. ..	<i>Nil</i>
18	Pumpkin .. ..	Traces
19	Garlic .. ..	"
20	Onion .. ..	"
21	Green banana pulp .. ..	<i>Nil</i>
22	<i>Rangalu</i> (sweet potato) .. ..	0
23	Squash .. ..	Traces
24	French beans .. ..	0.35-0.4
25	<i>Lau</i> (gourd) .. ..	0.16
26	Cucumber .. ..	0.175-0.2
27	Beet root .. ..	0
28	Cauliflower .. ..	Trace
29	Green mango (without skin) .. ..	"
30	Country peas (green) .. ..	1.94-2.0
31	Red pepper (green) .. ..	0.75-0.9
32	Carrot .. ..	2.0-5.6
33	<i>Kachu</i> ( <i>arum</i> ) .. ..	0

TABLE—concl'd.

Number.	Foodstuff.	Mg. carotene per 100 g. of material.
<i>ANIMAL FOODS—concl'd.</i>		
79	<i>Kôî</i> fish—flesh ..	Trace
	„ „ liver ..	„
80	<i>Rôhita</i> fish—flesh ..	0·06
	„ „ liver ..	1·22
81	Egg-white ..	0
82	Egg-yolk—hen ..	0·14
	„ duck ..	0·134

## STUDIES ON THE ABSORPTION OF CAROTENE AND VITAMIN A IN THE HUMAN SUBJECT.

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THE metabolic rôle of carotene and its efficacy as a pro-vitamin A in curing certain symptoms associated with vitamin-A deficiency in rats has been conclusively proved for some time. That it does play a similar part in human nutrition has special significance in India where carotene is the only source of this vitamin for most of the people. The necessity for investigating in the human subject, some at least of the factors which are concerned in its absorption, is hence of considerable importance.

While doing this experiment we also took the opportunity to follow the absorption of a commercial concentrate of vitamin A. This was of special interest in view of the fact that the vitamin was either free of its natural solvent or in an abnormal concentration in some fatty medium. Further, some clinicians have informed us that in many cases they have the impression that cod-liver oil as a therapeutic agent is more effective than the special preparation. The main object, however, of this investigation was to determine how far carotene in the form of two kinds of vegetables rich in this substance, namely, carrots and spinach, was absorbed on a typical Indian diet with and without fat. One of us (S. M. D. G.), a healthy Bengali male of 29 years of age and 147 lb. body-weight, acted as the experimental subject.

The general technique employed was that of the superimposition method which has been used for many years in metabolic balance experiments. A standard carotene-free diet was consumed during the whole course of the experiment which was divided into three periods; period I, standard diet alone (usually 4 days); period II, standard diet plus the food to be investigated (4 days); and period III, standard diet again (4 days). Another food could then be taken up making a fourth period if necessary. The faeces were collected in two-day periods and the excretion of carotene calculated therefrom. The excretion of carotene obtained in the basal period was then subtracted from the values for the experimental period in order to obtain the true figure for the unabsorbed carotene when the specific foodstuff was being ingested. The basal diet consumed was as follows:—

	Oz.
1. Rice (polished) .. .. .	16
2. Pulses: (lentils, <i>mung</i> , <i>arhar</i> ) .. .. .	2
3. Fish .. .. .	4
4. Vegetables: (potato, <i>patôl</i> , white turnip, gourd, pumpkin). .. .. .	4
5. Mustard oil .. .. .	$\frac{1}{2}$
6. <i>Ghee</i> .. .. .	1
7. Condiments, small quantities	

In experiments III, IV, and V the fat (*ghee* and mustard oil) was omitted. The carrots were ingested raw, while the spinach was cooked in water, which was afterwards consumed. The results are summarized in the Table. The carotene in the raw materials (carrot and spinach) was estimated as follows:—

TABLE.

Period.	Length of period (days).	Total amount of foodstuff ingested (g.).	Total amount of carotene ingested (mg.).	Total amount of carotene excreted (mg.).	Carr-Price reaction of the fecal extract.	Net total carotene absorbed (mg.).	Percentage carotene absorbed.
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*Experiment I.—CARROTS (ordinary basal diet).*

I	(a)	3	0	0	2.377	Negative	..	..
	(b)	2	0	0	0.631	Negative	..	..
II	(a)	3	170	16.40	4.10	Positive	} 21.28	77.83
	(b)	2	113	10.94	3.544	Faint		
III	(a)	2	0	0	2.08	Faint	..	..
	(b)	2	0	0	0.68	Negative	..	..
IV	(a)	2	320	30.91	4.724	} Strongly positive	} 53.78	86.99
	(b)	2	320	30.91	4.680			

TABLE—concl'd.

Period.	Length of period (days).	Total amount of foodstuff ingested (g.).	Total amount of carotene ingested (mg.).	Total amount of carotene excreted (mg.).	Carr-Price reaction of the faecal extract.	Net total carotene absorbed (mg.).	Percentage carotene absorbed.
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*Experiment II.—SPINACH (ordinary basal diet).*

I	(a)	2	0	0	0.454	Negative	..	..
	(b)	2	0	0	0.405	Negative	..	..
II	(a)	2	113	5.244	1.125	} Faint }	9.319	88.85
	(b)	2	113	5.244	0.954			
III	(a)	2	0	0	0.781	Faint	..	..
	(b)	2	0	0	0.402	Negative	..	..

*Experiment III.—SPINACH (fat-free basal diet).*

I	(a)	2	0	0	0.62	Faint	..	..
	(b)	2	0	0	0.56	Very faint	..	..
II	(a)	2	113	5.244	2.96	} Strongly positive }	5.488	52.32
	(b)	2	113	5.244	3.22			
III	(a)	2	0	0	0.81	Faint	..	..
	(b)	2	0	0	0.66	Very faint	..	..

*Experiment IV.—VITAMIN-A CONCENTRATE (ordinary basal diet).*

I	..	4	0	0	..	Negative	..	..
II	..	2	2.0	..	..	*Negative	..	..
III	..	2	4.0	..	..	*Negative	..	..

*Experiment V.—VITAMIN-A CONCENTRATE (fat-free basal diet).*

I	..	2	0	0	..	Negative	..	..
II	..	2	4.0	..	..	*Negative	..	..

\* In these cases a pink coloration was obtained with  $\text{SbCl}_3$  reagent.

The raw material was cut into slices or small pieces and freed from extraneous matter, etc. About two grammes of it was macerated with about half a gramme of sand and then successively extracted with ethyl alcohol, acetone and ether until no yellow coloration was obtained with the latter which is the main solvent. The ethereal layer was then washed free from alcohol, acetone, etc., and then saponified in the cold with 20 per cent ethyl-alcoholic potash for some 15 minutes in a separating funnel. The potash was washed off with water and the final ethereal solution was dried with fused sodium sulphate and evaporated to dryness. The residue was taken up with petroleum ether, washed with 60 per cent ethyl alcohol, dried with fused sodium sulphate and the solution made up to a known volume. The carotene was estimated calorimetrically by the Lovibond tintometer. By this method it was found that raw carrots contain about 9.66 mg. per hundred grammes and raw spinach about 4.6 mg. per cent of carotene.

In the case of the faeces, about 2 grammes to 3 grammes of it was macerated with an excess of fused sodium sulphate in a mortar and then the carotene extracted with ether. The procedure was then similar to that described above for the vegetables.

#### DISCUSSION.

It will be seen from the Table that the faeces extract gave a figure in yellow units although the Carr-Price reaction was negative. The nature of this substance, which, however, was present only in traces, is unknown. The figure obtained, however, expressed in yellow units, was subtracted from the values obtained during the experimental period when the reaction became positive showing that there was some excretion of carotene.

As regards the absorption of carotene, it will be noted—experiments I and II—that the retention is equally good with either carrots or spinach. The chemical state in which the substance is present in the vegetables is apparently favourable for an almost complete absorption provided fat is present in the diet. An increase in the quantity of carrots in the second part (period IV—experiment I) apparently has, at that level of intake, no adverse effect on the absorption, 15 mg. per day being absorbed as efficiently as 5 mg. or 6 mg. The effect of the presence or absence of fat in the diet on the absorption of carotene from spinach is clearly shown in experiments II and III. In experiment II with fat, out of 10.49 mg. ingested, 9.318 mg. were absorbed, while in experiment III, under identical conditions except for the absence of fat, only 5.488 were retained.

This observation may be of considerable importance in India where all over there tends to be a scarcity of fat and in certain areas where vegetables are scarce, this may be the decisive factor in determining the appearance of signs of deficiency disease.

*Absorption of vitamin A.*—Experiments IV and V give the results obtained when a commercial preparation of vitamin A was taken on a fat-free diet. The equivalent of 15,000 to 30,000 International Units of vitamin A were ingested in those experiments. The antimony trichloride test was done on an extract of faeces similar to that employed for the carotene estimation. In both experiments the test was negative. One can hence conclude that apart from bacterial decomposition in the intestine, these results favour the assumption that all the vitamin has been absorbed.



*The absorption of the isomers of carotene.*—The excretion of carotene in fairly large quantities in experiment III led the authors to examine in a preliminary way the nature of the carotene excreted. The total carotene was extracted from 785 g. faeces of period II—experiment III and the angle of rotation observed. The total carotene recovered was 6.18 mg. and the angle of rotation was  $0.25^\circ$ . The optical rotation of the *alpha* form is  $+326^\circ$  and that of *beta* nil (Strain, 1935). The amount of the *alpha* form in the solution is hence given by the equation :—

$$X = \frac{15 \times 0.25}{2 \times 326} = 5.75 \text{ mg.}$$

The percentage of *alpha* carotene excreted is hence 93.04, while that of the spinach ingested was only 24.4. Apparently on the fat-free diet the active form *beta* carotene has been almost completely and selectively absorbed. The validity of this observation is dependent on the absence in the faecal extract of dextro-rotatory substances other than *alpha* carotene. Further work is in progress on this point which is of considerable interest in view of the fact that the two isomers in their chemical reactions are almost identical.

#### CONCLUSIONS.

Carotene ingested by the human subject on a typical Bengali diet appears to be absorbed equally well, either when fed raw as carrots or cooked in the form of *sak* (spinach).

The percentage of absorption of carotene on a fat diet is about 90 in contrast to about 50 on the similar diet without fat. There is evidence to show that the body may preferentially absorb the physiologically active *beta* isomer of carotene.

There is evidence that a highly concentrated extract of vitamin A is, apart from bacterial decomposition, completely absorbed.

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## THE VITAMIN-B<sub>1</sub> CONTENT OF SOME COMMON INDIAN FOODSTUFFS.

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THE investigation recorded below covers the analysis of some 40 representative samples of common Indian foods for their vitamin-B<sub>1</sub> content. The number analysed is unfortunately not very large owing to the time involved in biological assays of this type. The data recorded, however, should be for the most part adequate to give an opinion on the vitamin-B<sub>1</sub> intake of the average Indian and above all Bengali consuming his accustomed diet. The method employed is that of the rat growth technique elaborated by Roscoe (1930). Young rats of 30 g. to 45 g. weight were put on the following B<sub>1</sub>-free diet:—

	Per cent.
Casein .. .. .	15
Dextrine .. .. .	65.5
Yeast autoclaved .. .. .	10.0
Butter fat .. .. .	2.5
Salt .. .. .	5
Cod-liver oil .. .. .	2

When the body-weight curve began to fall, a weighed quantity of the food to be investigated was given, care being taken to see that it was all consumed. In the case of the vegetables a small weighed piece of the sample was given the animal each day, while with the cereals a certain percentage of the dextrine was replaced by the cereals to be tested and the total food consumed each day obtained by weighing the special (Slonaker) food pan. The intake of cereal which produced the requisite growth could then be calculated.

The unit of B<sub>1</sub> was taken as that quantity which when given each day would produce an increase of weight of 10 g. per week for at least three weeks. Most of the foods were tested on at least 6 rats in order to find the quantity which would give as exactly as possible a 10 g. increase. In some cases where the average increase was a little more or less than 10 g. per week a calculation of the vitamin units was made from a graph of Coward *et al.* (1933). This method in most cases appeared to give results which agreed with the figures obtained when a growth of exactly 10 g. per week was obtained. The results are all expressed as units of vitamin B<sub>1</sub> per 100 g. food. This unit (Roscoe) is equal to an International Unit which is equivalent to 4 micrograms of the Jansen and Donath crystals. The investigation covered vegetables and cereals and a glance at figures (*see* Table) will show that the vegetables are all relatively poor in B<sub>1</sub>, the number of units varying from 11 to 40 per 100 g. The cereals, however, are all comparatively rich in this accessory foodstuff and particular attention should be drawn to green *mung*, *arhar* dal, lentils and beans. The mean value for all cereals is round about 150 with the exception of polished rice which has only some 26 units per 100 g. in contrast to atta with 110. As rice or atta form the staple background of diets in India the significance of the results needs no stressing. The superiority of the dals is off-set to a certain extent by the fact that they are not and cannot be consumed in such quantities as rice or atta.

TABLE.

Number.	Foodstuff.	Units of vitamin B <sub>1</sub> per 100 g. of material.
VEGETABLES.—		
1	<i>Patól</i> .. ..	16-20
2	Brinjal .. ..	19-20
3	<i>Karela</i> (bitter gourd) ..	22-25
4	Tomato .. ..	22-24
5	<i>Jhinga</i> (ridge gourd) ..	20-23
6	<i>Lal sak</i> .. ..	10-13
7	Banana (green), without skin ..	12-17
8	Potato .. ..	20-30
9	<i>Kachu</i> (arum) .. ..	22-25
10	<i>Kumra</i> (pumpkin) .. ..	18-24
11	Peas (green) .. ..	20-25
12	Spinach .. ..	18-20
13	<i>Lau</i> (gourd) .. ..	19-20
14	Cabbage .. ..	20-25

TABLE—concl'd.

Number.	Foodstuff.	Units of vitamin B <sub>1</sub> per 100 g. of material.
VEGETABLES—concl'd.		
15	Turnip .. ..	12-14
16	Cauliflower .. ..	15-20
17	Squash .. ..	17-18
18	Carrot .. ..	20-24
19	Cucumber .. ..	18-20
20	Bhindi (lady's fingers) ..	20-22
21	Rangalu (sweet potato) ..	11-13
22	Kalmi sak (ipomœa) ..	25-30
23	French beans .. ..	25-26
24	Puin sak .. ..	21-39
25	Papaya (green) .. ..	25-30
26	Radish .. ..	30
27	Onion .. ..	16
CEREALS.—		
28	Gram .. ..	90-96
29	Green mung .. ..	150-160
30	Black mung .. ..	140
31	Sona mung .. ..	115
32	Lentils .. ..	150
33	Maize .. ..	70
34	Arhar .. ..	190
35	Beans .. ..	150-170
36	Wheat (atta) .. ..	100-110
37	Millet (bajri) .. ..	110
38	Barley .. ..	150
39	Polished*rice .. ..	26
40	Peas .. ..	150

It is not intended to discuss the vitamin-B<sub>1</sub> requirements of man, as a paper based on diet survey on this subject is already projected and will be published later. It should be mentioned, however, that from the data given by Cowgill (1934) the amount of vitamin-B<sub>1</sub> units to protect from beri-beri is assessed at about 150 to 350. This, however, is a protective dose and as yet we have no knowledge as to the optimum requirements of man for this substance.

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NUTRITIONAL SURVEY OF SCHOOLBOYS IN CALCUTTA  
AND THE PUNJAB BY MEANS OF CLINICAL  
OBSERVATIONS, A. C. H. INDEX OF  
NUTRITION AND OTHER  
MEASUREMENTS.

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THE criticism of diets as adequate or inadequate in the light of their chemical composition, useful though it may be, must ultimately be correlated with the health of the community subsisting on such diets. The investigation recorded in this paper is intended as an attempt to find standards of nutritional status and the incidence of malnutrition among the children in the areas concerned. The fields of study cover some 3,000 boys of varying economic classes and of different communities in Calcutta and 1,250 of more or less the same economic class but of different 'communities' in Ferozepore (Punjab). The original object of the investigation was to test out a special method for estimating defective development or malnutrition called the Arm-Chest-Hip index of nutrition (henceforth A. C. H. index). This method devised by Doctors Franzen and Palmer of the American Child Health Association consists essentially of the following :—

A. C. H. INDEX.

The original purpose of the measurements and the ratios obtained by this index was to select out children who should be subjected to further medical examination for defects or disease. The originators devised the technique from a selection of a number of different skeletal and muscular measurements, finally

adopting on the basis of their constancy and significance the three mentioned, namely: arm, chest, and hip. All measurements are made in centimetres. The arm girth is measured by the maximum girth of the biceps (*a*) when the arm is hanging down and (*b*) when the tip of the fingers are touching the shoulder, care being taken to avoid marked contraction (making) of the biceps. The two readings are then summed. The chest depth is measured antero-posteriorly at the nipple with a pair of sliding callipers. The readings at normal inspiration and expiration are taken and summed. The hip width is measured with the same callipers from the heads of the trochanters, the subject standing with the heels and toes together. The difference between the sum total arm girth and that of the chest depth is found, and from the tables it is noted that with a certain hip width this difference should be above a certain figure. If it is less than the standard figure in the table the child is selected for further medical attention. The particular set of figures employed depends on how rigid a selection of defectives is desired. It should be noted that the method does not select all who should be subjected to further medical examination, and vice versa others are taken who may be quite normal. The term *selected* has been employed by the originators to designate those who are below an arbitrary physical standard.

In general, it would appear that the essential aim of the method is to show whether the child has an adequate proportion of soft tissue to skeleton. In order to find out the applicability of the method in this country, the height and weight were taken and observations on certain signs of deficiency disease and other abnormalities were looked for. As far as Calcutta is concerned, the scope of the inquiry widened as the investigation proceeded. All the children were examined for (*a*) phrynoderma (toad-skin), noted by Nicholls (1934) in Ceylon and others elsewhere in the tropics; the condition appears to be a deficiency disease, the exact ætiology of which is unknown, (*b*) angular stomatitis, a similar condition with an unknown ætiology, and (*c*) Bitot's spots due to vitamin-A deficiency.

During the course of this survey it was noted that the incidence of enlarged tonsils and carious teeth appeared to be high, so data on these points were collected from then on. Recently, Nicholls (1936) has described certain conditions called malocclusion and displaced teeth which he has found to be correlated with economic status. This unfortunately up to the present has not been recorded. One of us (H. E. C. W.), however, was struck early in the investigation in Calcutta by the number of children with unsymmetrical or displaced teeth. Data are now being kept on this point. The tongue was always observed but nothing abnormal was noted. Few cases of obvious anæmia were seen. The data collected in the Punjab cover the three communities: Hindu, Mohammedan, and Sikh, all more or less of the lower middle class of shopkeepers, artisans, etc. Observations on the skin, eyes, teeth, etc., were made similarly to what had been done in Calcutta. The observer (B. A.) of this particular survey, however, does not wish to stress the eye or skin condition, as he has not had experience in those conditions. The absence of any marked positive case would appear to indicate, however, that they are not common.

#### CALCUTTA AREA RESULTS.

The most striking points to note about the children of the same community but of different economic class is the contrast between their heights and weights



(see Figs. 1 to 8 and Tables I to IX). With the exception of the Marwari community, the Hindus, Mohammedans, and Anglo-Indians all showed this difference. The Marwaris are a partial exception, as the poorer class are probably Hindustanis and not completely vegetarian. This fact may hence account to a certain degree for the greater average height of the poorer class. Both classes were, however, of about the same weight, a feature which may be partially accounted for by the fact that the poorer classes do manual labour. The fact remains that the poor Marwaris have less muscle tissue in proportion to height than the others.

### *Incidence of disease or other defects.*

The incidence of conditions such as toad-skin and Bitot's spots is for all practical purposes *nil*. Only one questionable case of toad-skin was seen and no active corneal ulceration was recorded. The milder degrees of Bitot's spots are probably easily missed, but, if there had been any tendency to such conditions, it is to be expected that more advanced cases would have been observed. The two conditions which showed a marked correlation with economic status were the carious teeth and enlarged tonsils. The most marked instance is that of the Marwari community (see Tables VI and VII) where the figures for the incidence of both enlarged tonsils and caries together in one child are 23 per cent for the poor and only 8.3 per cent for the better-off class. It should be pointed out, however, that enlarged tonsils may not be by any means due directly to nutritional defects; local hygiene, overcrowding, etc., may all play a part. A similar correlation between economic status and those two conditions singly or combined is found in all the communities, the highest incidence being 56 per cent for caries among the poor Anglo-Indians. This condition is in all probability partially associated with a deficiency of calcium in the diet. In favour of this is the observation that the lowest figure of all is among the Marwaris (21 per cent), who consume milk and atta both of which are good sources of minerals. The high figure for the Anglo-Indians, 56 per cent, is probably associated with a low milk intake and the consumption of white bread and rice without atta. It is unlikely, however, that the calcium intake is the only factor concerned as no cases of rickets were noted.

### *A. C. H. index.*

The American investigators chose standard figures for the index so that 10 per cent of the children would be selected as below par as compared with the others. In Calcutta, using those standards, differences in the percentages selected in the different groups examined have been found but no positive correlation between height, weight, or economic status on the one hand and the index figures on the other have been noted. The figures for the better-off Marwaris, Bengali Hindus, and Bengali Mohammedans are 4.1 per cent, 16.3 per cent, and 19.8 per cent, while the corresponding figures for the poor classes are 1.0 per cent, 1.5 per cent, and 2.8 per cent, respectively. No correlation of the index with the incidence of such conditions as caries, enlarged tonsils, or even underweight has been observed. As regards the Anglo-Indian community, the percentage selected in both economic classes is as low as 3.7. This seems the more remarkable in view of the fact that almost certainly the lower class children must have been of poorer physique

FIG. 1

# CALCUTTA

British -----  
 Rich Bengali Hindus. ---●---  
 Poor " " ---○---

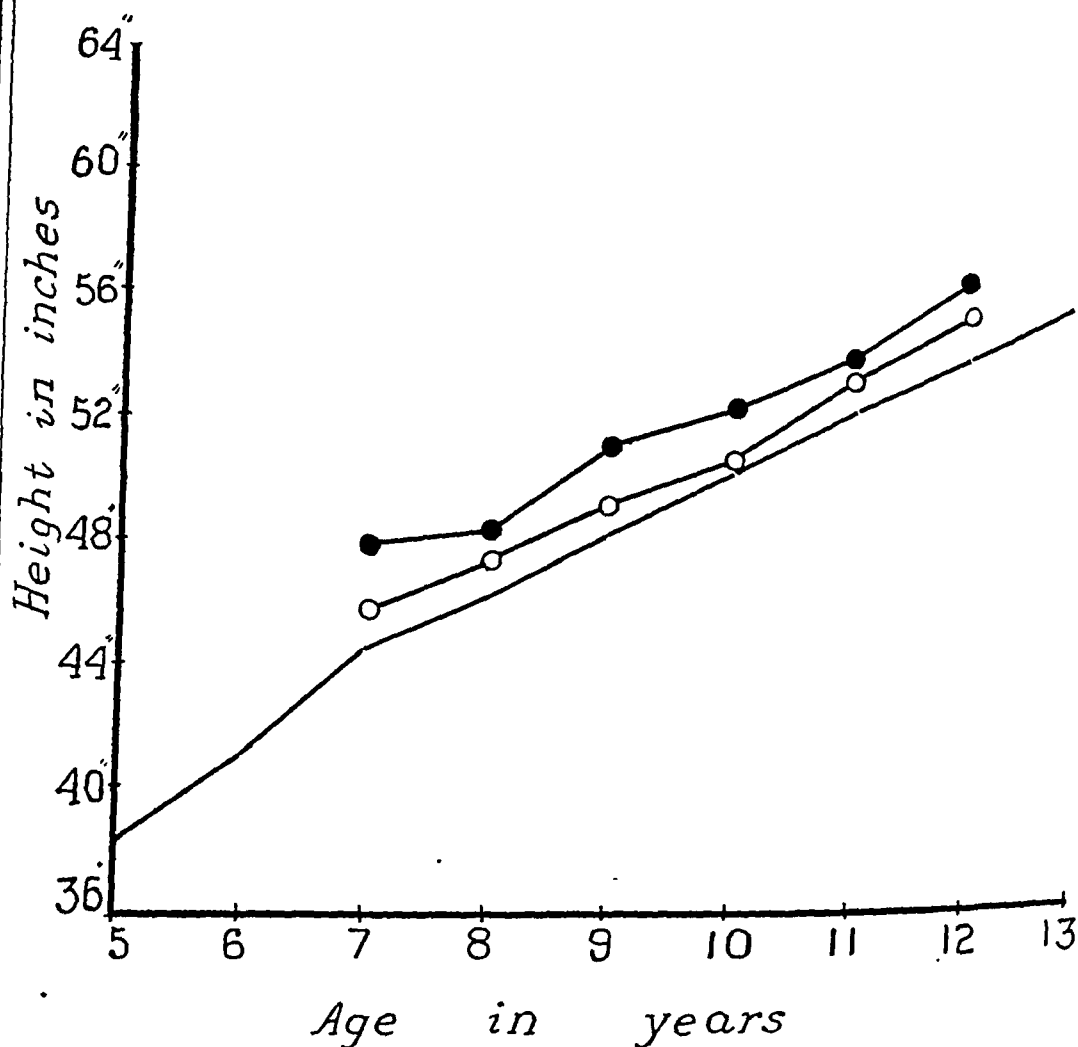
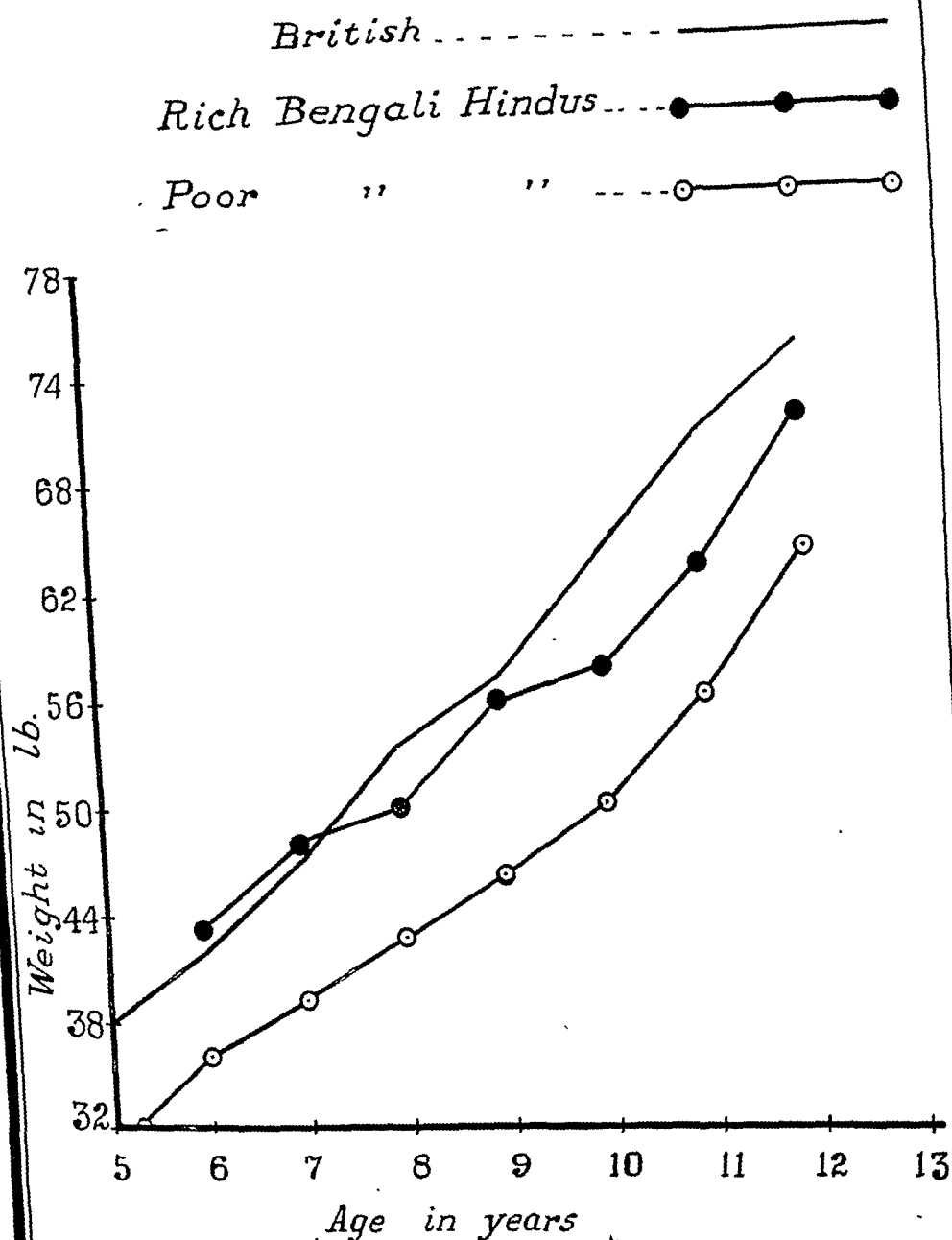


FIG. 2.

# CALCUTTA





# CALCUTTA

FIG. 4.

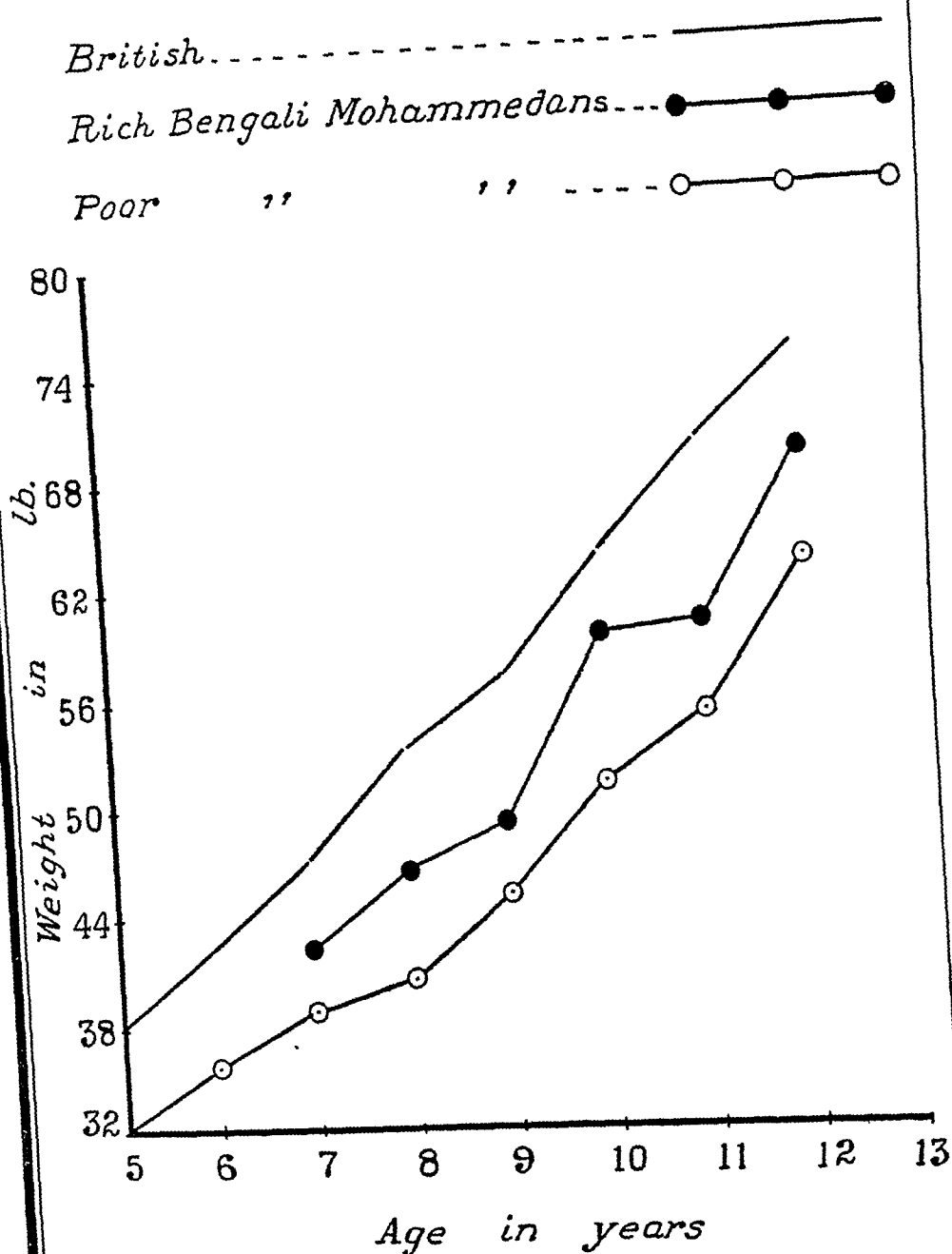


FIG. 5. CALCUTTA

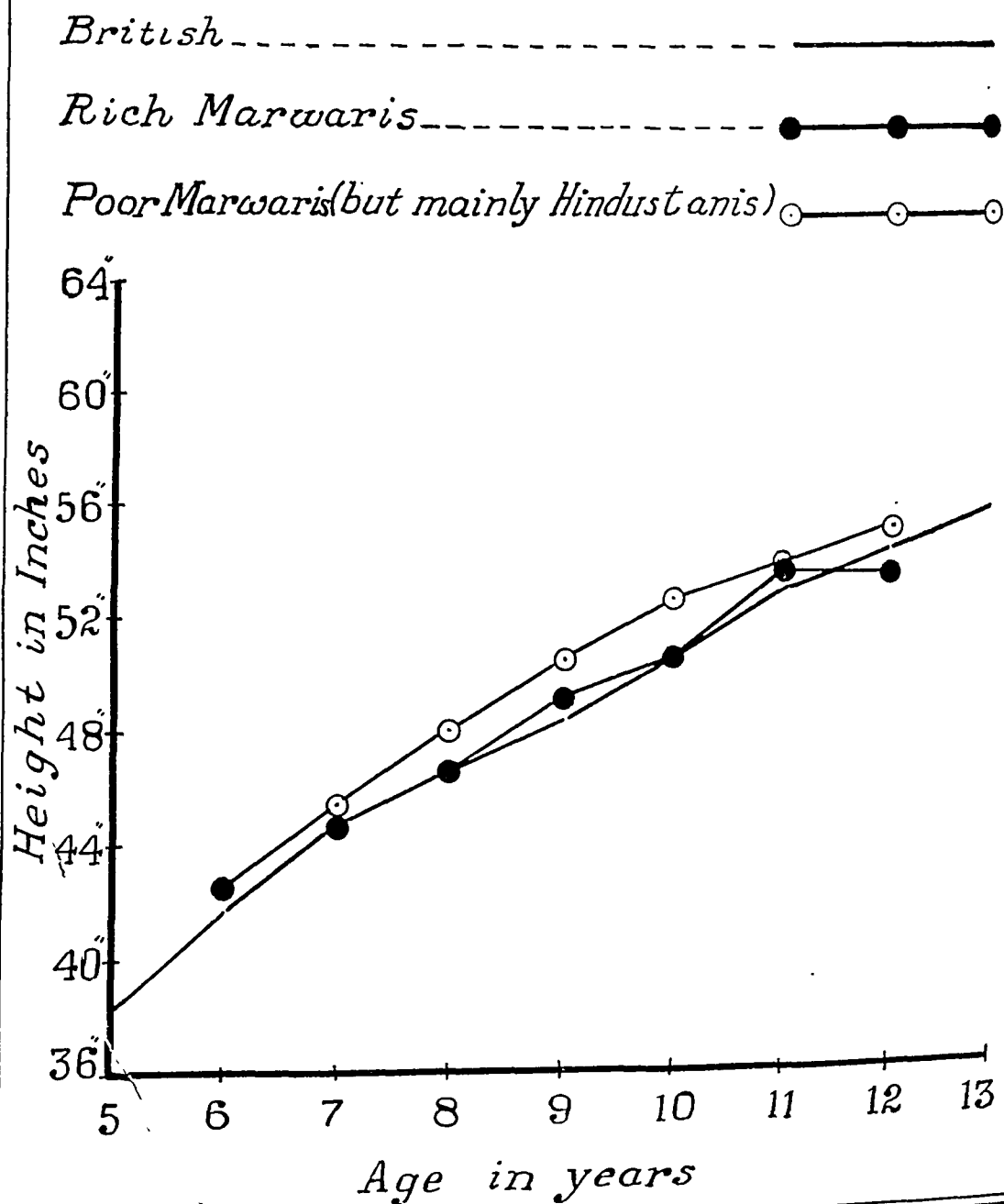
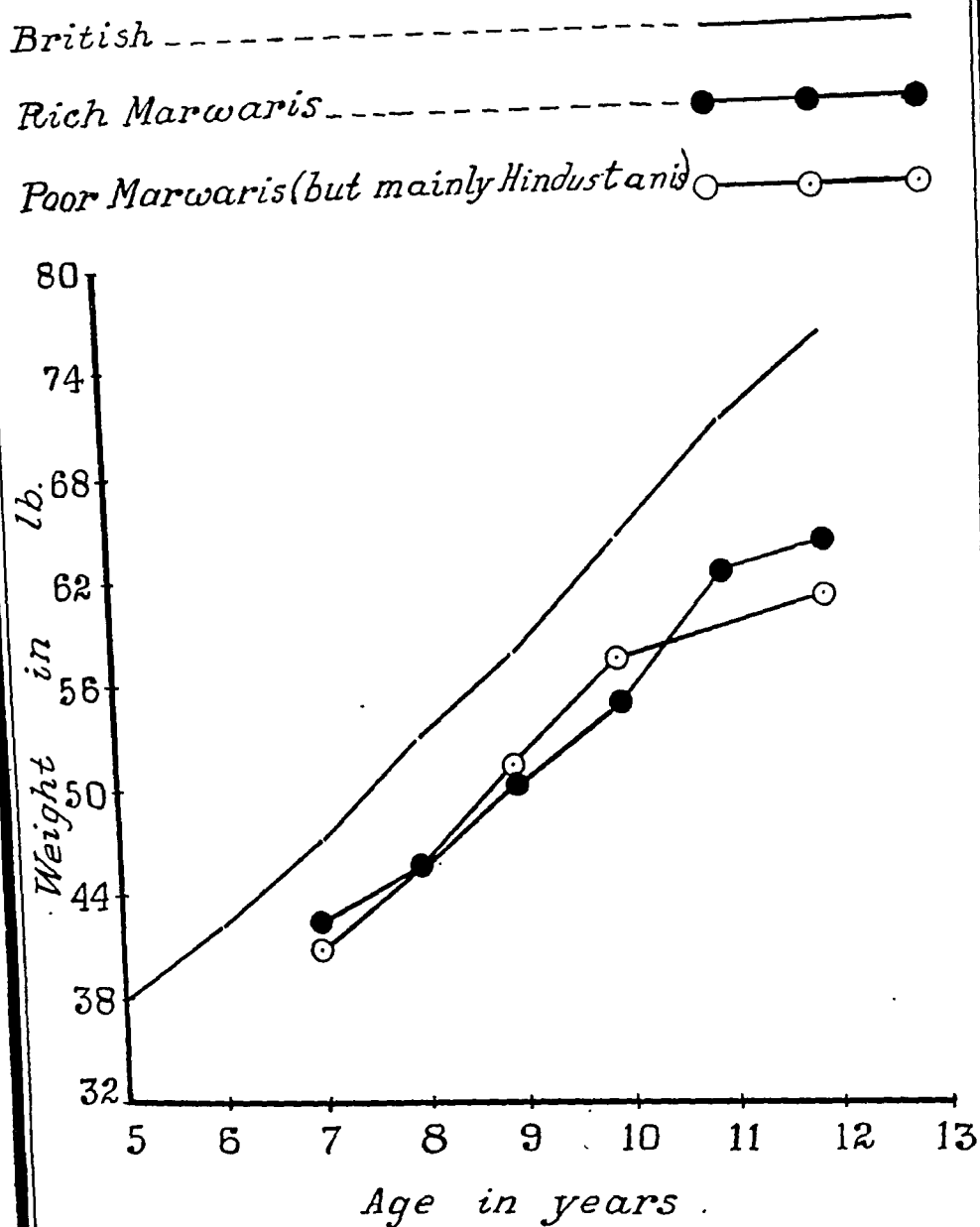


FIG. 6.

## CALCUTTA



# CALCUTTA

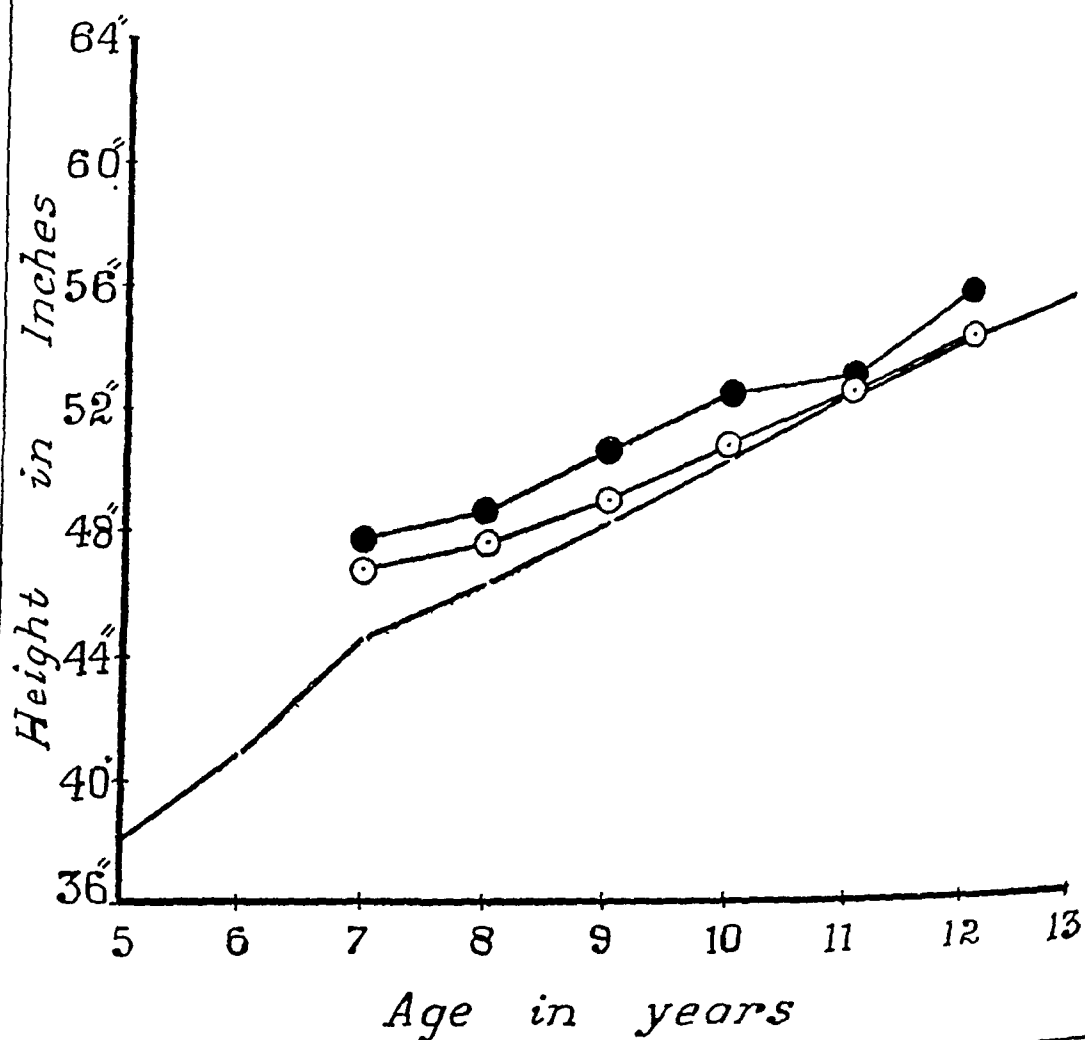
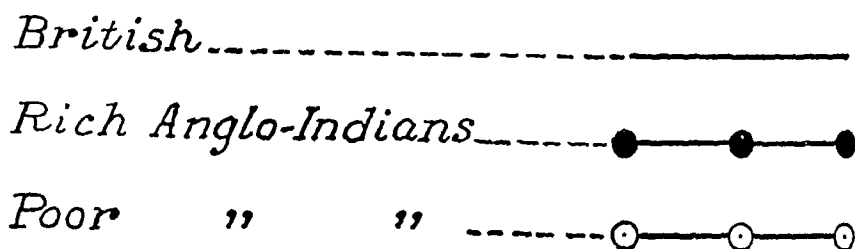
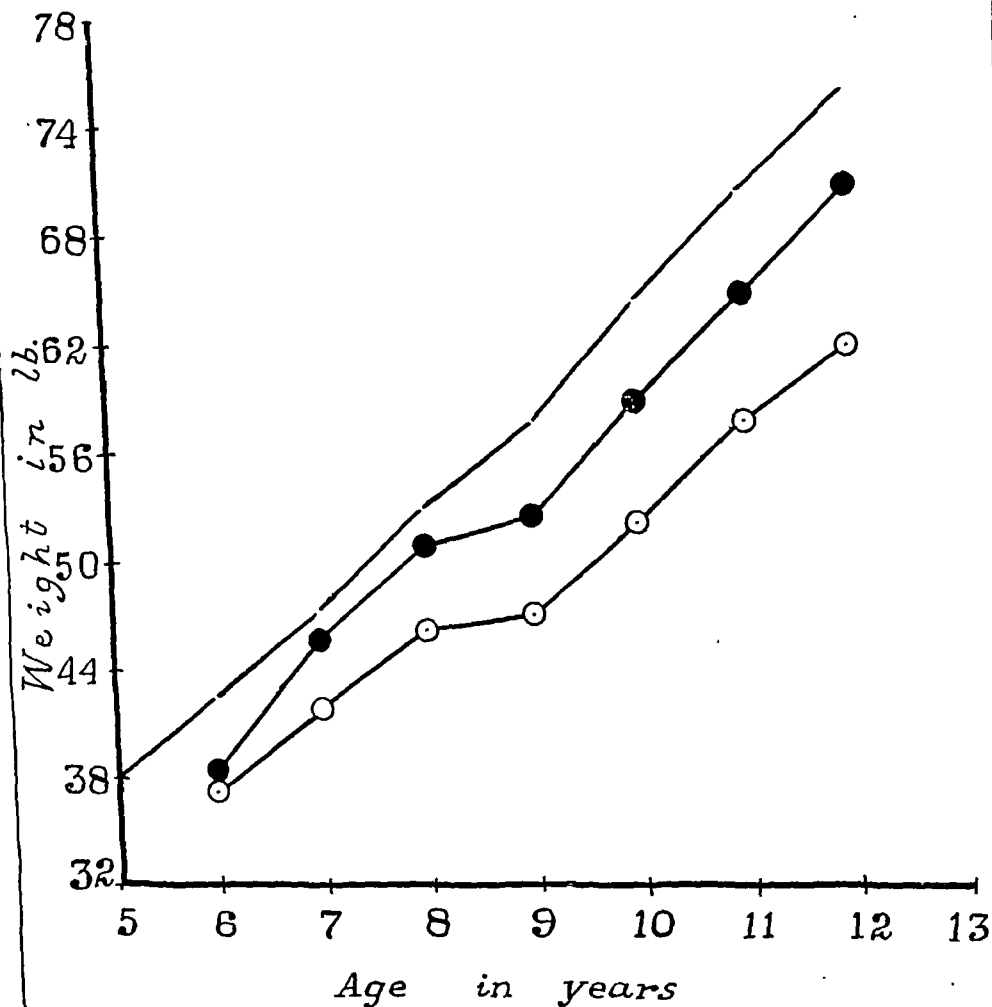




FIG. 8

# CALCUTTA

*British* -----  
*Rich Anglo-Indians* ----- ● ----- ● ----- ●  
*Poor* " " ----- ○ ----- ○ ----- ○



than their American brethren and yet *a priori* racially and hence physically should not differ from them so much as might the pure Indian. In other words, a selection of at least 10 per cent might have been expected among this community. More work will certainly have to be done on this subject particularly in regard to finding standards applicable to India itself.

#### PUNJAB INVESTIGATION RESULTS.

The 1,250 children of the three communities as mentioned above were all of the same economic class and in addition were in mixed schools. It would appear likely that the money available for food was approximately the same for all and that the difference in diet was probably not so marked as it might be elsewhere. All communities (B. A.) consumed flesh within the restrictions imposed by their beliefs and it would appear that a difference in diet was not very marked.

Before commencing the measurements, the children of each school were lined up and divided into four groups according to their physique, namely, good, fair, moderate, and poor. This arbitrary classification proved to be very significant.

#### *Height and weight.*

The heights and weights of the three communities do not show any particular difference beyond the racial one which is noted in the increase in rate of growth and increase of weight of the Sikhs at about 11 and 12 years of age (Figs. 9 and 10).

#### *Incidence of disease.*

The incidence of caries varies between 30 per cent and 38 per cent, the Mohammedans representing the smaller figure which is approximately the same as that found among the poor members of this community in Calcutta, viz., 27 per cent. The figures for enlarged tonsils are approximately the same for all communities ranging between 24 per cent and 27 per cent and approaches what is found among the better classes (excluding Anglo-Indian) in Calcutta. As this was a rural area without overcrowding and real urban conditions, the local hygienic environment may have accounted for this feature.

#### *A. C. H. index.*

The index in this particular area shows in contrast to Calcutta (see Table X) a definite positive correlation with the clinical assessment of physique. The percentages selection among the three communities, Hindu, Mohammedan, and Sikh, are 5.6, 10.1, and 6.9 respectively. If, however, only those children assessed as of poor physique are taken for analysis, the percentages selected are 28.7, 30, and 40, respectively, for the three communities. Further, if only those children who declared that they consumed milk regularly are subjected to analysis, again a positive correlation is noted. This difference is most marked in the case of the Mohammedans where the total selection is 10.1 per cent, while of those taking milk the corresponding figure is only 1.3 per cent.

Here as in Calcutta there is no consistent correlation between underweight and the incidence of caries or enlarged tonsils and the index.

PUNJAB

FIG. 9.

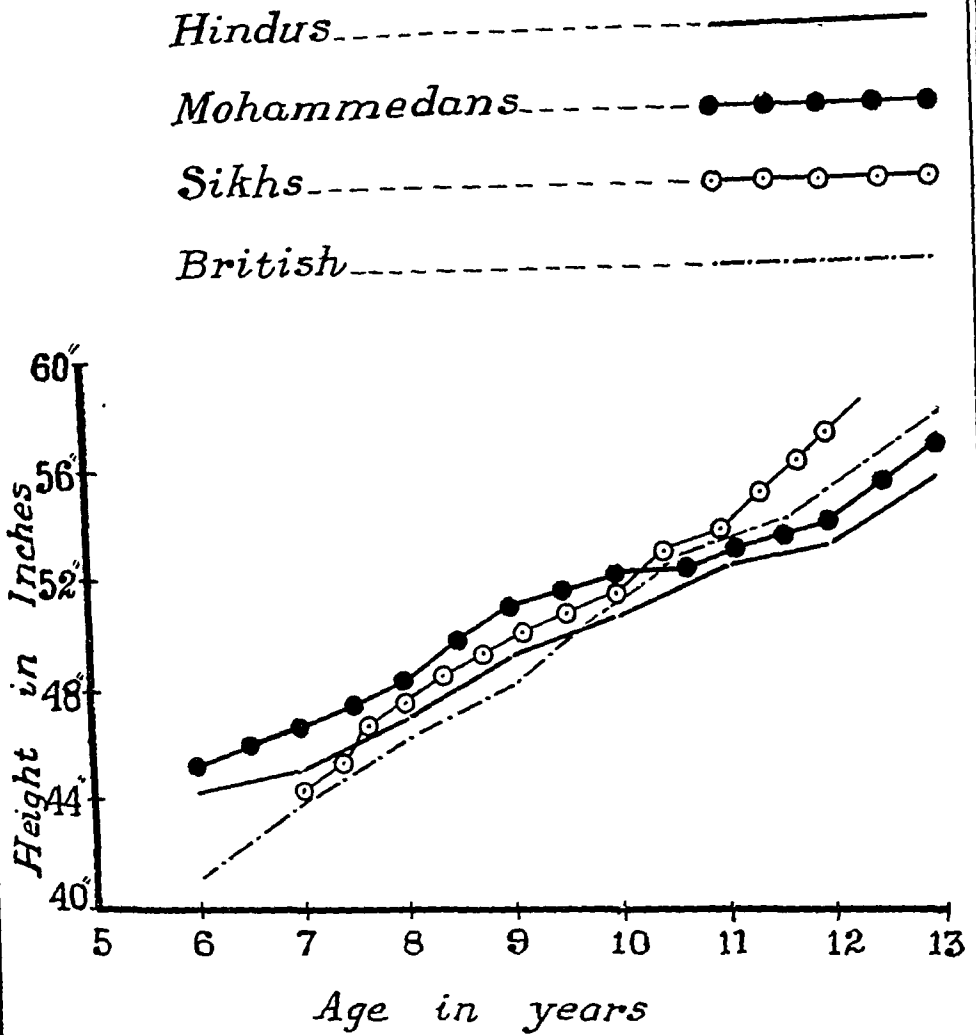


FIG. 10

## PUNJAB

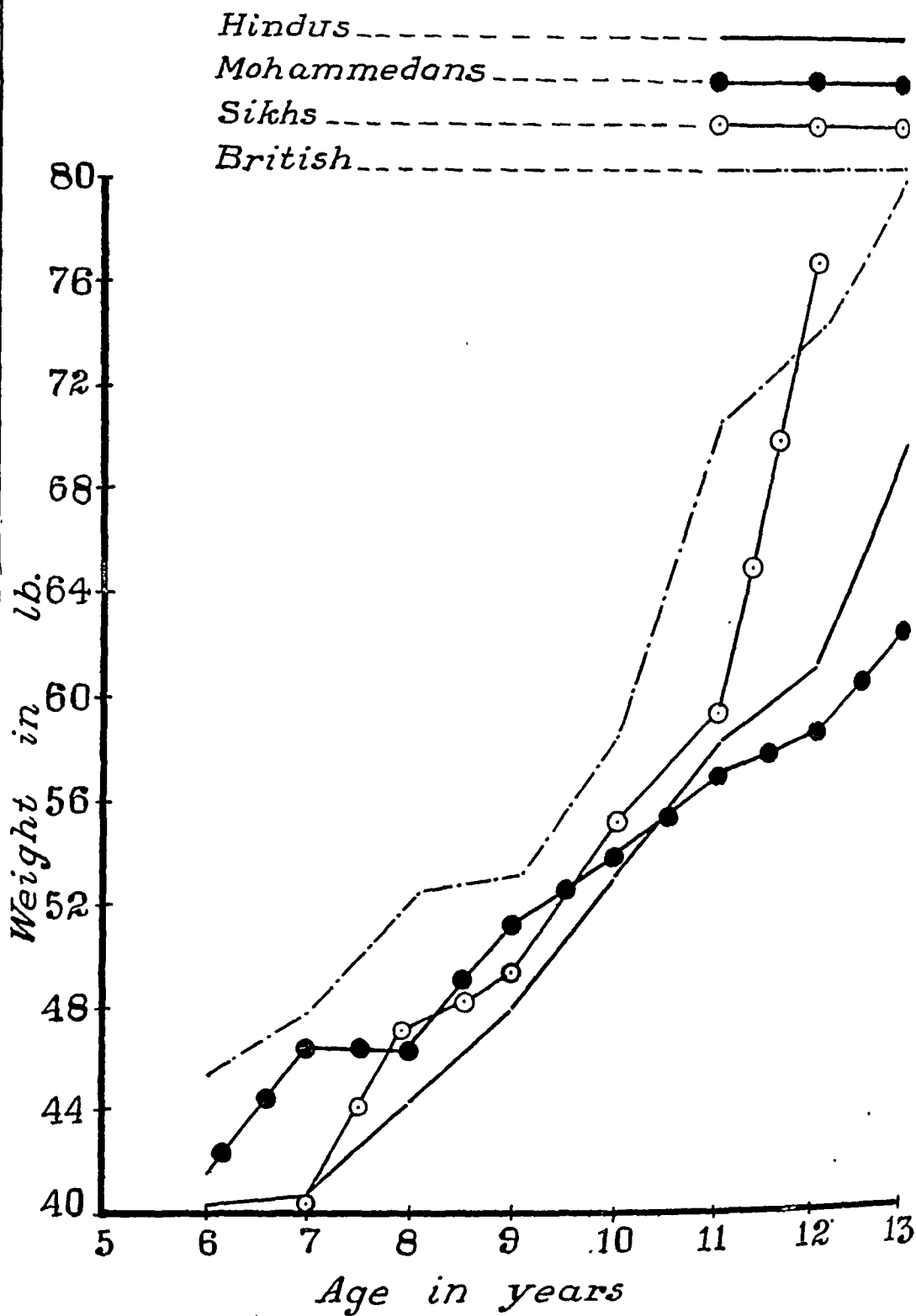


TABLE I.

*Rich class Bengali Hindu boys.*

SCHOOLS A AND B.					SCHOOL B.	
Age group.	Number of boys.	Average height in inches.	Average weight in lb.	Number selected by A. C. H. index.	Number of boys.	Caries number.
7	15	48.07	47.71	0	9	7
8	28	48.55	50.20	6	16	3
9	57	51.34	56.19	7	28	11
10	77	52.44	58.31	17	27	9
11	80	53.85	63.40	9	54	12
12	74	56.51	75.04	15	50	6
TOTALS ..	331	..	..	54 (16.3 per cent)	184	48 (26.1 per cent)

TABLE II:

*Middle class Bengali Hindu boys.*

SCHOOLS C, D, AND E.					SCHOOL C.	
Age group.	Number of boys.	Average height in inches.	Average weight in lb.	Number selected by A. C. H. index.	Number of boys.	Caries number.
7	28	45.63	42.43	1	18	8
8	64	47.40	44.97	4	39	15
9	84	49.18	49.36	8	38	13
10	127	51.10	53.44	16	79	26
11	110	52.89	59.18	19	68	19
12	88	54.78	64.58	19	47	10
TOTALS ..	501	..	..	67 (13.3 per cent)	289	91 (31.4 per cent)

TABLE III.

*Poor class Bengali Hindu boys.*

Age group.	Number of boys.	Average height in inches.	Average weight in lb.	Number selected by A. C. H. index.	CARIES.		ENLARGED TONSILS.		ENLARGED TONSILS + CARIES.	
					Number.	Per cent.	Number.	Per cent.	Number.	Per cent.
7	114	45.43	39.19	0	56	50.0	51	44.7	9	7.8
8	140	47.55	43.45	1	72	51.0	55	32.1	14	10.0
9	104	48.85	46.34	1	41	39.4	41	39.4	7	6.7
10	121	50.63	50.20	5	45	37.1	60	49.9	12	9.9
11	87	53.15	56.23	3	26	29.8	33	37.9	7	8.0
12	32	55.75	64.30	1	7	21.8	19	59.3	3	9.3
TOTALS ..	498	..	..	11 (1.5 per cent)	247	43	259	42	50	8.6

TABLE IV.

*Rich class Bengali Mohammedan boys.*

Age group.	Number of boys.	Average height in inches.	Average weight in lb.	Number selected by A. C. H. index.	Caries number.	Enlarged tonsils number.	Enlarged tonsils + caries number.
7	2	46.65	42.87	0	2	1	1
8	5	46.55	46.20	0	2	1	1
9	12	50.55	49.40	1	6	2	0
10	34	53.03	59.03	2	5	12	2
11	30	54.50	60.85	7	9	6	3
12	33	57.02	69.37	13	2	8	1
TOTALS ..	116	..	..	23 (19.8 per cent)	26 (22.4 per cent)	30 (25.8 per cent)	8 (6.9 per cent)

TABLE V.

*Poor class Bengali Mohammedan boys.*

Age group.	Number of boys.	Average height in inches.	Average weight in lb.	Number selected by A. C. H. index.	Caries number.	Enlarged tonsils number.	Enlarged tonsils + caries number.
7	58	45.15	39.10	1	17	33	14
8	40	43.39	40.80	0	15	21	7
9	42	48.95	45.70	2	10	17	2
10	45	50.84	50.78	1	15	13	4
11	37	52.62	55.45	1	8	10	4
12	28	55.97	62.88	2	4	6	1
TOTALS ..	250	..	..	7 (2.8 per cent)	69 (27.6 per cent)	100 (40 per cent)	32 (12.8 per cent)

TABLE VI.

*Rich class Marwari boys.*

Age groups.	Number of boys.	Average height in inches.	Average weight in lb.	Number selected by A. C. H. index.	Caries number.	Enlarged tonsils number.	Enlarged tonsils + caries number.
7	14	44.75	41.60	0	4	3	0
8	17	46.55	45.20	0	8	6	3
9	14	49.05	50.20	0	4	3	1
10	48	50.90	54.93	1	8	7	2
11	69	53.30	62.11	5	13	19	4
12	79	53.85	64.30	4	16	24	10
TOTALS ..	241	..	..	10 (4.1 per cent)	53 (21.9 per cent)	62 (25.7 per cent)	20 (8.3 per cent)

TABLE VII.

*Poor class Marwari boys.*

Age group.	Number of boys.	Average height in inches.	Average weight in lb.	Number selected by A. C. H. index.	Caries number.	Enlarged tonsils number.	Enlarged tonsils + caries number.
7	29	45.77	40.33	0	6	17	5
8	33	48.25	45.57	1	19	21	15
9	31	50.54	51.62	0	12	9	5
10	32	52.75	56.86	0	8	14	4
11	4	57.05	70.50	0	0	2	0
12	9	54.78	61.75	0	4	3	3
TOTALS ..	138	..	..	..	49 (35.5 per cent)	66 (47.8 per cent)	32 (23.1 per cent)



TABLE VIII.

*Rich class Anglo-Indian boys.*

Age group.	Number of boys.	Average height in inches.	Average weight in lb.	Number selected by A. C. H. index.	Caries number.
7	8	47.71	45.81	0	3
8	17	48.53	50.60	1	8
9	26	50.45	52.70	0	16
10	31	52.48	58.03	2	12
11	31	52.70	64.27	1	8
12	29	56.15	70.00	1	8
TOTALS ..	142	..	..	5 (3.5 per cent)	55 (38.7 per cent)

TABLE IX.

*Poor class Anglo-Indian boys.*

Age group.	Number of boys.	Average height in inches.	Average weight in lb.	Number selected by A. C. H. index.	Caries number.
7	6	47.25	42.3	0	4
8	26	47.95	45.9	0	20
9	18	48.88	46.5	2	10
10	23	50.75	52.2	0	14
11	22	52.65	57.41	0	8
12	11	54.35	61.6	2	4
TOTALS ..	106	..	..	4 (3.7 per cent)	60 (56.6 per cent)

TABLE X.

*Analysis of Ferozepore (Punjab) data.*

Community.	Number of children.	Enlarged tonsils per cent.	Caries per cent.	PER CENT SELECTED BY A. C. H. INDEX FROM					
				Total group.	Good physique.	Fair physique.	Moderate physique.	Poor physique.	Those taking milk.
Punjab Hindus ..	642	26.2	38.3	5.6	4.4	5.0	2.9	28.7	4.1
Punjab Mohammedans ..	465	24.9	30.5	10.1	14.8	8.6	7.8	30.5	1.3
Punjab Sikhs ..	144	27.0	36.8	6.9	8.6	0	4.4	40.0	5.7

TABLE XI.

*Analysis of Calcutta data.*

Community.	Enlarged tonsils per cent.	Caries per cent.	Enlarged tonsils and caries per cent.	Number selected by A. C. H. index.
Bengali Hindus : Rich class ..	..	26.2	..	16.3
„ „ Middle class ..	..	31.4	..	13.3
„ „ Poor class ..	42.0	43.0	8.6	1.5
„ Mohammedans : Rich class ..	25.8	22.4	6.9	19.8
„ „ Poor class ..	46.0	27.6	12.8	2.8
Marwaris : Rich class ..	25.7	21.9	8.3	4.1
„ (Hindustani) : Poor class	47.8	35.5	23.1	1.0
Anglo-Indians : Rich class ..	..	38.7	..	3.5
„ „ Poor class ..	..	56.6	..	3.7

## DISCUSSION.

Taken all over, the children examined in Calcutta and Ferozepore (Punjab) do not show any signs, as far as present clinical experience goes, of malnutrition due to a gross lack of balance in the diet, particularly in regard to the protective foods. That a defective diet exists, however, in the Calcutta children is shown only too clearly in the heights and weights of the different economic classes of each community. In Ferozepore, if the clinical assessment of the children, taken in conjunction with the higher figure for those selected by the index among those children arbitrarily assessed as of poor physique, is correct, the incidence of malnutrition may be no higher than would have been selected in the U. S. A. It should be pointed out also that the lower figure given by the index among those taking milk regularly, favours the assumption that the physique might be better were the diet improved.

As regards the nature of the deficiency in Calcutta, the observations recorded here, when considered along with our knowledge of the diets consumed in the town, would seem to warrant the conclusion that the main deficiency is one of protein primarily and fat to a certain degree. Experience in Calcutta shows that a deficiency of calories is not marked among the Indian communities but in the poor Anglo-Indian class there was strong evidence obtained from home conditions that there was often a gross lack in the quantity of food, even bread, consumed. As far as the observation extends in Calcutta, the A. C. H. index does not appear to be applicable. Apparently those of the poorer classes are built on an all-round smaller scale, so that there is no disproportion between muscle and bone. It is for this reason that we incline to the view that lack of the proximate principle protein is the main deficiency. It is also likely that a special set of standard figures will have to be made for the index, if it is to apply to India, and possibly also for the different races. The negative findings with the index in Calcutta, however, in view of what it purports to measure and in view also of the fact that malnutrition does exist, have been of value, in our opinion, in suggesting or rather emphasizing a deficiency, namely protein, which was observed and commented on many years ago by McKay (1912) in this town.

In view of the fact that protein is an essential building material for body tissue without which, even in the presence of an abundance of vitamins, normal growth could not be completed, it appears to us advisable to stress this point particularly in propaganda work where the lay public possibly tend to think of the vitamins to the exclusion of other nutriment. It should also be pointed out that in many cases the exact vitamin requirements can be expressed in quantitative terms, whereas no such figure has yet (based on any objective criterion) been given for protein. A general agreement has been arrived at as a safe or optimum protein intake but there can be no doubt that the majority of the people of India have a protein consumption which falls short of any authoritative standard for the foodstuff.

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| NICHOLLS, L. (1934) | .. | .. | <i>Ind. Med. Gaz.</i> , <b>69</b> , p. 241. |
| <i>Idem</i> (1936)  | .. | .. | <i>Ceyl. Jour. Sci.</i> , <b>4</b> , p. 1.  |

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Community.	Number of children.	Enlarged tonsils per cent.	Caries per cent.	PER CENT SELECTED BY A. C. H. INDEX FROM					
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„ „ Middle class ..	..	31.4	..	13.3
„ „ Poor class ..	42.0	43.0	8.6	1.5
„ Mohammedans: Rich class ..	25.8	22.4	6.9	19.8
„ „ Poor class ..	46.0	27.6	12.8	2.8
Marwaris: Rich class ..	25.7	21.9	8.3	4.1
„ (Hindustani): Poor class	47.8	35.5	23.1	1.0
Anglo-Indians: Rich class ..	..	38.7	..	3.5
„ „ Poor class ..	..	56.6	..	3.7

## ASCORBIC-ACID OXIDASE IN PLANT AND ANIMAL TISSUES.

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SZENT-GYÖRGYI (1930, 1931) has shown that cabbage leaf contains an enzyme which oxidizes ascorbic acid at a very high rate. Tauber, Kleiner and Mishkind (1935) have obtained concentrated ascorbic-acid oxidase preparations from Hubbard squash. The present piece of work was undertaken in order to obtain information about the general distribution of the enzyme in various fruits, vegetables and animal tissues, necessary for the further investigation of the enzyme.

### EXPERIMENTAL.

#### I. Plant tissues.

The fruits and the vegetables with which the experiments were performed were all obtained from the local market. Four or five fruits or vegetables of each variety were cut up into pieces, mixed up and 20 g. from the sample were taken, ground well in a mortar with a small amount of sea-sand, shaken gently for 5 minutes with 60 c.c. of 30 per cent alcohol in a 500 c.c. flask and filtered and the filtrate was used as the source of the enzyme (see Tauber, Kleiner and Mishkind, *loc. cit.*).

In order to test for the enzyme in the alcoholic extract, the solutions were taken in the manner mentioned below in three conical flasks of the same capacity provided with rubber corks:—

- (1) Ten c.c. of the alcoholic extract + 3 c.c. of ascorbic-acid solution (containing a known amount of ascorbic acid) + 7 c.c. of acetate buffer of pH 5.6.

(2) Ten c.c. of the alcoholic extract + 10 c.c. of the buffer of pH 5.6.

(3) Three c.c. of ascorbic-acid solution [same solution as that used in flask (1)] + 6 c.c. of 50 per cent alcohol + 11 c.c. of the buffer of pH 5.6.

No. (1) represents the enzyme solution with ascorbic acid; No. (2) provides the control with the enzyme solution without ascorbic acid; and No. (3) provides the control with ascorbic acid without the enzyme. The total volume is 20 c.c. in each case and contains the same percentage of alcohol.

The flasks were incubated in a thermostat at 38°C. for 45 minutes in all experiments. After the period of incubation, they were taken out of the thermostat, and 1 c.c. of a 2 per cent sulphuric-acid solution was added to each of the flasks in order to stop the activity of the enzyme, if any. Then the amount of ascorbic acid in each of the flasks was estimated by the usual titrimetric technique with the indophenol indicator (Ghosh and Guha, 1935). The total amount of ascorbic acid present in flasks (2) and (3) *minus* the amount of ascorbic acid present in flask (1) after incubation gives the amount of ascorbic acid oxidized by the enzyme.

Figures are given in the Table.

## II. Animal tissues.

A known weight of animal tissue (usually 14 g. to 16 g.) was taken in a mortar, ground well with sea-sand, shaken gently for 5 minutes with 30 per cent alcohol in a flask and then centrifuged. The centrifugate was used as the source of the enzyme. For 1 g. tissue, 3 c.c. of the 30 per cent alcohol were used. The amount of ascorbic acid taken was 3 mg. in a total volume of 20 c.c. in each case.

TABLE.

Serial number.	Bengali names.	English names.	Botanical names.	Amount of ascorbic acid taken (in mg.).	Amount of ascorbic acid (in mg.) oxidized by the enzyme under stated conditions.
1	Anarash ..	Pineapple ..	<i>Ananas sativa</i> ..	3.0	0
2	Peara .. ..	Guava ..	<i>Spidium gujava</i> ..	3.0	0
3	Mung (not germinated).	Black gram ..	<i>Phaseolus mungo</i> ..	3.0	0
4	Chhola (not germinated).	Bengal gram ..	<i>Cicer arietinum</i> ..	3.0	0
5	Dhenrôs ..	Lady's fingers..	<i>Hibiscus esculentus</i> ..	2.0	0
6	Peauj .. ..	Onion ..	<i>Allium cepa</i> ..	3.0	0
7	Rasun ..	Garlic ..	<i>Allium sativum</i> ..	1.2	0

TABLE—contd.

Serial number.	Bengali names.	English names.	Botanical names.	Amount of ascorbic acid taken (in mg.).	Amount of ascorbic acid (in mg.) oxidized by the enzyme under stated conditions.
8	Mula .. ..	Radish ..	<i>Raphanus sativus</i> ..	3.0	0
9	Matar-shuti ..	Peas (green) ..	<i>Pisum sativum</i> ..	2.0	0
10	Shalgom ..	Turnip ..	<i>Brassica campestris</i> , var.	2.0	0
11	Tomato ..	Tomato ..	<i>Lycopersicum esculentum</i>	2.0	0
12	Batapi lebu ..	Shaddock ..	<i>Citrus decumana</i> ..	1.5	0
13	Lebu ..	Lemon ..	<i>Citrus medica</i> ..	1.5	0
14	Kamala lebu ..	Orange ..	<i>Citrus aurantium</i> ..	1.5	0
15	Paniphal ..	Water chestnut	<i>Trapa bispinosa</i> ..	1.8	0
16	Ata ..	Custard apple..	<i>Anona squamosa</i> ..	1.8	0
17	Pepe (green) ..	Papaya ..	<i>Carica papaya</i> ..	1.5	0
18	Tal ..	Palm ..	<i>Borassus flabellifer</i> ..	1.8	0
19	Am ..	Mango ..	<i>Mangifera indica</i> ..	3.0	0
20	Narikel ..	Coco-nut ..	<i>Cocus nucifera</i> ..	1.8	0
21	Bhutta ..	Maize ..	<i>Zea mays</i> ..	1.8	0
22	Angur ..	Grape ..	<i>Vitis vinifera</i> ..	1.2	0.10
23	Chhola (germinated)	Bengal gram ..	<i>Cicer arietinum</i> ..	1.5	0.20
24	Palong sak ..	Spinach ..	<i>Spinach oleracea</i> ..	3.0	0.23
25	Bel ..	Wood apple ..	<i>Aegle marmelos</i> ..	3.0	0.25
26	Kancha lanka ..	Green chillies..	<i>Capsicus indicus</i> ..	3.0	0.28
27	Puin sak ..	— ..	<i>Bassela cardifolia</i> ..	2.0	0.32
28	Apel ..	Apple ..	<i>Pyrus molus</i> ..	3.0	0.38
29	Kala (Singapuri) ..	Banana ..	<i>Musa sapientum</i> ..	2.0	0.38
30	Kala (Martaman) ..	Banana ..	<i>Musa sapientum</i> ..	2.0	0.38
31	French beans ..	French beans..	<i>Phaseolus vulgaris</i> ..	3.0	0.42
32	Nespati ..	Pears (country)	<i>Pyrus communis</i> ..	3.0	0.57

TABLE—concl'd.

Serial number.	Bengali names.	English names.	Botanical names.	Amount of ascorbic acid taken (in mg.).	Amount of ascorbic acid (in mg.) oxidized by the enzyme under stated conditions.
33	Dalim .. ..	Pomegranate ..	<i>Punica granatum</i> ..	1·8	0·66
34	Aloo .. ..	Potato ..	<i>Solanum tuberosum</i> ..	3·0	1·16
35	Kalmi sak ..	Ipomœa ..	<i>Ipomœa reptans</i> ..	2·0	1·27
36	Mung (germinated)	Gram ..	<i>Phaseolus mungo</i> ..	3·0	1·41
37	Begoon ..	Brinjal ..	<i>Solanum melongena</i> ..	3·0	1·46
38	Fulkopi ..	Cauliflower ..	<i>Brassica oleracea</i> , var. <i>botrytis</i> .	3·0	1·59
39	Misti kumro ..	Sweet gourd ..	<i>Cucurbita maxima</i> ..	3·0	1·84
40	Gajar .. ..	Carrot ..	<i>Daucus carota</i> ..	3·0	1·86
41	Patôl .. ..	— ..	<i>Trichonasthes dioica</i> ..	3·0	1·91
42	Jhinge ..	Ridge gourd ..	<i>Luffa acutangula</i> ..	3·8	2·04
43	Lettuce ..	Lettuce ..	<i>Lactuca sativa</i> ..	3·0	2·22
44	Lau .. ..	Gourd ..	<i>Lagenaria vulgaris</i> ..	3·0	2·29
45	Hinche ..	— ..	<i>Enhydra fluctuans</i> ..	3·0	2·34
		White gourd ..	<i>Benincasacriapra</i> ..	3·0	2·76
		Cucumb. ..	<i>Cucumis sativus</i> ..	5·0	2·94

— were obtained from the local slaughter house and the  
 .. Pineapple 3 to 6 hours after slaughtering of the animal. The  
 .. Guava obtained by killing them in the laboratory and the  
 .. immediately. The fish-liver was obtained from the  
 ermi- Black  
 .. ts with p<sup>l</sup> animal tissues, a known weight of  
 ermi- Benga solution his was diluted to a definite volume  
 .. frigerat<sup>r</sup> phuretted hydrogen was passed into  
 .. Lady's e any y oxidized ascorbic acid which might  
 .. sol phuretted hydrogen was driven off by  
 .. Onion  
 .. Garlic s  
 .. s in any of the animal tissues investigated,  
 .. w, guinea-pig, rabbit and fowl; the liver



tissue of the *katla* fish (*Catla catla*) and the kidney tissue of the rat; the adrenal, pituitary, brain and pancreas of the cow.

### DISCUSSION.

In this work we have not made a strictly quantitative comparison among the substances investigated, as the concentration of ascorbic acid used was not constant in all experiments and conditions with reference to the presence of metallic catalysts and other interfering substances, which might influence the oxidation of ascorbic acid, could not possibly be the same. Attempts will be made later to fix the conditions for more quantitative comparisons. The results indicate, however, that among the foodstuffs investigated, Sasha (*Cucumis sativus*), Chal kumro (*Benincasacriapra*), Hincbe (*Enhydra fluctuans*), Lau (*Lagenaria vulgaris*), Lettuce (*Lactuca sativa*), Jhinge (*Luffa acutangula*), Patôl (*Trichonasthes dioica*), Gajar (*Daucus carota*), Misti kumro (*Cucurbita maxima*), Fulkopi (*Brassica oleracea*, var. *Botrytis*) and Begoon (*Solenum melongena*) contain a fair amount of ascorbic-acid oxidase, Sasha (*Cucumis sativus*) and Chal kumro (*Benincasacriapra*) being the richest sources among these. It is interesting to note that germination produces ascorbic-acid oxidase in Kancha mung (*Phaseolus mungo*) just as it produces ascorbic acid.

In the liver and kidney of different species, such as the cow, guinea-pig, rabbit, fish, and fowl, no ascorbic-acid oxidase could be detected. The adrenal, pituitary, brain and pancreas of the cow were also examined but none of them contained the enzyme. These results are in marked contrast to those obtained with plant tissues many of which contain fair amounts of the oxidase.

### SUMMARY.

(1) A large number of plant and animal tissues have been investigated with reference to their contents of ascorbic-acid oxidase.

(2) Among these Sasha (*Cucumis sativus*) seems to be the richest source of the enzyme.

(3) Germination leads to the formation of ascorbic-acid oxidase in Kancha mung (*Phaseolus mungo*).

(4) The animal tissues investigated, in contrast to most of the plant tissues, seem to be completely devoid of the enzyme.

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animals were estimated and also 30, 60, 90, and 120 minutes after the injection of glucose. The figures are shown in Table I.

- (b) The experiments were performed in the same way as before. This group of animals received only an injection of 0.5 c.c. adrenalin chloride. The results of the estimation of blood sugar and calcium are shown in Table II. There was apparently an increase in the sugar content of the blood but no change in the calcium content.
- (c) This group was also treated in the same way as group (a), except that adrenalin was injected immediately after the injection of glucose. The results of estimation of blood sugar and calcium have been shown in Table III. It is seen that, while there was a rise in sugar, there was no change in the calcium content of the blood.
- (d) This group of animals was also treated in the same way as above except that adrenalin chloride was injected half an hour after glucose. The figures for blood calcium and sugar are given in Table IV.
- (e) This group of animals was also treated as in the case of the other groups but they received an injection of adrenalin half an hour previous to the administration of glucose. The results of the estimation are to be found in Table V. In these animals there was no rise of calcium.

It was noticed that the administration of glucose was followed by an increase of titrable alkali of blood. For titration 1.5 c.c. of serum of the treated animal was diluted with 1.5 c.c. of distilled water and titrated with N/50 HCl using cresol red as indicator. This increase of titrable alkali has been shown in Tables I and IV.

Injections of alkali, however, did not increase the calcium content of blood.

The result of the experiments may be summarized in the following table:—

	Calcium content of blood.	Glucose content of blood.	Titrable alkali in blood.
1. Adrenalin injection.	No change.	Increase.	Not investigated.
2. Adrenalin and glucose injected successively.	„	Marked increase.	No change.
3. Glucose and adrenalin injected successively.	„	Increase.	„
4. Adrenalin injected half an hour after glucose.	Increase in calcium but after adrenalin is injected it is restored to normal.	„	Increase at the beginning but later comes down to normal along with calcium.
5. Alkali solution injected in a small quantity.	No change.	No change.	No perceptible change.

TABLE I.

Weight of rabbits in g.	AFTER INJECTION OF GLUCOSE.															
	BEFORE INJECTION.				30 MINUTES.			60 MINUTES.			90 MINUTES.			120 MINUTES.		
	Doses of glucose Injection in c.c.	Calcium in mg. per cent.	Sugar per cent.	Alkali in c.c.	Calcium in mg. per cent.	Sugar per cent.	Alkali in c.c.	Calcium in mg. per cent.	Sugar per cent.	Alkali in c.c.	Calcium in mg. per cent.	Sugar per cent.	Alkali in c.c.	Calcium in mg. per cent.	Sugar per cent.	Alkali in c.c.
985 ..	0.9	12.0	0.066	1.05	16.0	0.111	1.20	16.0	0.117	1.20	16.5	0.117	1.20	16.0	0.166	1.20
1,360 ..	1.3	16.0	0.086	1.20	16.0	0.133	1.20	18.0	0.153	1.35	18.5	0.181	1.35	18.0	0.181	1.35
1,115 ..	1.1	11.2	0.080	1.20	12.5	0.111	1.35	13.2	0.117	1.35	13.2	0.181	1.35	14.0	0.200	1.35
1,125 ..	1.1	12.0	0.090	1.05	13.5	0.133	1.20	14.0	0.181	1.35	16.0	0.200	1.50	16.0	0.200	1.50
1,320 ..	1.3	11.0	0.083	0.90	11.5	0.133	1.05	12.0	0.166	1.05	14.2	0.166	1.05	15.0	0.181	1.20
1,400 ..	1.4	11.2	0.076	0.90	11.0	0.105	0.90	13.2	0.117	1.05	14.0	0.117	1.20	14.5	0.166	1.20
1,168 ..	1.1	11.5	0.074	0.90	11.5	0.105	0.90	14.2	0.111	1.05	15.0	0.166	1.05	15.0	0.166	1.20
1,210 ..	1.2	12.0	0.080	0.90	14.0	0.117	1.05	16.0	0.117	1.20	15.8	0.133	1.20	15.5	0.181	1.20

TABLE II.

BEFORE INJECTION.			AFTER INJECTION OF ADRENALIN.							
			30 MINUTES.		60 MINUTES.		90 MINUTES.		120 MINUTES.	
			Calcium in mg. per cent.	Sugar per cent.	Calcium in mg. per cent.	Sugar per cent.	Calcium in mg. per cent.	Sugar per cent.	Calcium in mg. per cent.	Sugar per cent.
1	14.0	0.086	13.9	0.125	14.1	0.181	14.0	0.200	14.0	0.250
2	12.0	0.080	12.2	0.086	12.0	0.125	12.1	0.133	12.0	0.181
3	13.0	0.083	13.0	0.086	13.0	0.105	12.9	0.125	13.0	0.181
4	17.0	0.105	17.0	0.125	17.1	0.181	17.0	0.200	17.0	0.250
5	14.0	0.080	14.2	0.090	14.0	0.133	14.0	0.166	14.0	0.181
6	12.0	0.083	12.0	0.095	12.1	0.105	12.0	0.133	12.0	0.153
7	13.2	0.086	13.0	0.117	13.2	0.153	13.0	0.181	13.0	0.250
8	14.0	0.083	14.0	0.100	14.0	0.125	14.2	0.142	14.2	0.166

TABLE III.

Weight of rabbits in g.	BEFORE INJECTION.			SUGAR INJECTION IMMEDIATELY FOLLOWED BY ADRENALIN.							
				30 MINUTES.		60 MINUTES.		90 MINUTES.		120 MINUTES.	
	Doses of glucose injection in c.c.	Calcium in mg. per cent.	Sugar per cent.	Calcium in mg. per cent.	Sugar per cent.	Calcium in mg. per cent.	Sugar per cent.	Calcium in mg. per cent.	Sugar per cent.	Calcium in mg. per cent.	Sugar per cent.
1,368 ..	1.3	16.0	0.083	16.0	0.090	15.9	0.105	16.0	0.181	16.0	0.200
1,062 ..	1.0	11.2	0.086	11.2	0.100	11.2	0.153	11.0	0.181	11.2	0.250
1,120 ..	1.1	13.0	0.074	13.0	0.100	13.1	0.133	13.0	0.166	13.0	0.181
1,420 ..	1.4	12.2	0.076	12.0	0.105	12.0	0.153	12.2	0.181	12.2	0.200
1,400 ..	1.4	12.0	0.080	12.0	0.117	12.2	0.166	12.0	0.200	12.0	0.250
1,170 ..	1.1	13.0	0.083	13.0	0.133	13.0	0.181	13.0	0.181	13.0	0.200
1,210 ..	1.2	16.2	0.076	16.2	0.111	16.2	0.181	16.2	0.200	16.2	0.250
1,420 ..	1.4	11.5	0.080	11.5	0.117	11.5	0.153	11.5	0.181	11.5	0.200

TABLE IV.

Weight of rabbits in g.	BEFORE INJECTION.				30 MINUTES AFTER GLUCOSE INJECTION.			INJECTION OF ADRENALIN AFTER 30 MINUTES OF GLUCOSE INJECTION.					
	Doses of glucose injection in c.c.	Calcium in mg. per cent.	Alkali in c.c.	Sugar per cent.	Calcium in mg. per cent.	Alkali in c.c.	Sugar per cent.	30 MINUTES.			60 MINUTES.		
								Calcium in mg. per cent.	Alkali in c.c.	Sugar per cent.	Calcium in mg. per cent.	Alkali in c.c.	Sugar per cent.
1,210 ..	1.2	16.0	0.90	0.065	18.0	1.05	0.166	18.0	1.05	0.166	16.0	0.90	0.181
1,300 ..	1.3	14.0	1.05	0.090	16.6	1.20	0.111	16.0	1.20	0.166	15.0	1.05	0.200
1,000 ..	1.0	13.5	0.90	0.086	16.6	0.90	0.166	14.5	1.05	0.181	14.0	1.05	0.200
900 ..	0.9	12.0	0.90	0.074	14.5	1.20	0.111	13.5	1.05	0.153	13.5	0.90	0.181
1,540 ..	1.5	14.0	1.20	0.083	16.0	1.20	0.166	15.2	1.20	0.181	14.0	1.20	0.200
1,500 ..	1.5	15.0	0.90	0.080	17.0	1.05	0.111	15.0	0.90	0.153	15.0	1.05	0.166
980 ..	0.9	12.0	1.05	0.086	16.0	1.05	0.133	14.0	0.90	0.143	14.0	1.05	0.181
1,400 ..	1.4	11.0	0.90	0.076	14.0	1.05	0.133	14.0	0.90	0.153	13.0	1.05	0.200

TABLE V.

Weight of rabbits in g.	BEFORE INJECTION.		ADRENALIN INJECTION FOLLOWED BY GLUCOSE AFTER AN INTERVAL OF 30 MINUTES.			
	Doses of glucose injection in c.c.	Calcium in mg. per cent.	30 MINUTES Calcium in mg. per cent.	60 MINUTES Calcium in mg. per cent.	90 MINUTES Calcium in mg. per cent.	120 MINUTES Calcium in mg. per cent.
910 ..	0.9	12.0	12.2	11.9	12.0	12.0
1,220 ..	1.2	12.5	12.5	12.15	12.3	12.5
1,200 ..	1.2	11.0	11.2	11.0	11.2	11.0
1,110 ..	1.1	14.0	14.2	14.0	14.1	14.0
1,020 ..	1.0	12.0	12.1	12.0	12.0	12.0
1,050 ..	1.0	13.0	13.0	13.1	13.0	13.0
1,115 ..	1.1	12.0	12.0	12.0	12.0	12.0
1,120 ..	1.1	11.1	11.0	11.0	11.0	11.0

TABLE VI.

Weight of rabbits in g.	BEFORE INJECTION.			AFTER INJECTION OF GLUCOSE 30 MINUTES.	
	Doses of injection in c.c.	Phosphate in mg. per cent.	Calcium in mg. per cent.	Phosphate in mg. per cent.	Calcium in mg. per cent.
1,120 ..	1.1	5.0	12.0	4.1	14.0
1,010 ..	1.0	4.2	11.5	3.8	16.0
1,020 ..	1.0	4.0	13.0	3.5	14.2
1,510 ..	1.5	4.0	12.0	4.0	12.5
1,035 ..	1.0	3.5	11.2	2.8	14.0
1,260 ..	1.2	4.0	11.0	3.2	14.0
1,361 ..	1.3	3.0	12.0	3.0	12.5

## DISCUSSION.

It is evident from the above results that adrenalin exercises an inhibitory action on the increase of blood calcium after administration of glucose intravenously. It has been shown by various workers that the calcium content of blood is normally regulated by the hormone of parathyroid, vitamin D, and the phosphates of blood (Read, 1928). The influence of vitamin D in the increase of calcium in our experiments can be discarded, as all the animals were given the same food. The phosphates of blood shown in Table VI were found to have decreased whenever there was increase in calcium.



We know that increase of glucose in blood in all normal conditions of the organism stimulates increased insulin secretion. Again, pancreas and parathyroids are known to co-operate with one another regarding various metabolic activities of the body. It may, therefore, be assumed that glucose causes an increase of blood calcium through the influence of pancreas on parathyroids. Adrenal glands and pancreas are antagonistic to each other (Harrower, 1933). Consequently excess of adrenalin in blood may cause inhibition of pancreatic activity. This fact is corroborated by our experiments. It is clear thus that if adrenalin is injected along with glucose or immediately before or after glucose, glucose will not be allowed to exert its influence on pancreas owing to the inhibiting influence of adrenalin on pancreas, and hence there is no rise of calcium. From the above experiments it may be assumed that glucose has a stimulating action on parathyroids either directly or indirectly through pancreas.

Possible factors involved in increasing the alkalinity after administration of glucose :—

It was noticed that glucose increased the calcium content and alkalinity of blood simultaneously as shown in our experiments.

This increase of alkalinity may be attributed to the increase in the bases of blood. Administration of parathyroid also produces a temporary increase in the alkalinity of blood (Cameron, 1933). It is also possible, therefore, that glucose injection directly or indirectly stimulates parathyroid—thus increasing the alkalinity of blood. It is difficult to ascertain definitely which of these two factors is responsible for the increased alkalinity or whether both of these factors are involved in the process.

#### SUMMARY.

- (1) Glucose injection increases the calcium content of blood.
- (2) Adrenalin injection increases the blood sugar without any change in the calcium content.
- (3) Sugar injection immediately followed by adrenalin does not increase the calcium though blood sugar increases to a great extent.
- (4) If adrenalin be injected after a rise of calcium on glucose injection the curve of calcium gradually comes down to normal.
- (5) If glucose be injected after adrenalin no change of the calcium content takes place.

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## HÆMATOLOGICAL STUDIES IN INDIANS.

### Part VI.

#### INVESTIGATIONS IN 100 CASES OF MARKED ANÆMIA AMONGST TEA-GARDEN COOLIES.

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AN investigation into the cause of the anæmia that is so prevalent amongst tea-garden coolies was commenced by the writers in 1933. Some of the preliminary steps in this investigation, hæmatological examinations in normal city-dwelling Indians and in so-called 'normal' coolies, have been reported in the earlier parts of these studies.

In this paper, we are reporting the blood findings in definite cases of anæmia amongst the labour forces in gardens in the practices of Doctors D. Manson and G. Macdonald. The first series of cases were investigated during the cold weather months of 1933-1934, and the second between November 1934 and May 1935

#### THE FIRST SERIES.

*The clinical material.*—Our patients were untreated patients attending the hospitals on certain tea estates in the practices of the two doctors referred to above; most of these patients had been selected during the systematic examination of coolies carried out each year on these estates, but a few had reported sick voluntarily. The selection was made mainly on clinical grounds, that is to say, on appearance or because they complained of some shortness of breath, but a Tallqvist reading of 50 per cent, or less, was taken as the criterion. The clinical features presented by

the patients were fairly constant but do not lend themselves to analysis. Their general appearance was anæmic, with a slight degree of puffiness of the face and the whole body, and œdema of the legs. In a few cases there was obvious general anasarca, but usually the puffiness of the skin on the trunk was only noticeable after prolonged pressure, e.g., with the stethoscope. The tongue had the wash-leather appearance, but was never red or sore. Most of the patients were thin but not emaciated.

There was usually a hæmic murmur; the lungs were usually normal. The pulse rate was between 90 and 100 in the majority of cases, and the blood pressure low, seldom above 110 mm. Hg. (systolic). There were no constant intestinal symptoms and no nervous symptoms.

Night-blindness was a common complaint but there were few other obvious signs of specific nutritional deficiency, though there was evidence of general under-nourishment. Koilonychia was not noticed.

They were nearly all outdoor labourers, both men and women. None of them were aboriginal inhabitants of Assam, but some had been born on the gardens, nearly all had been in Assam for many years, and none had lived there for less than a year.

They came originally from different parts of India, and were members of innumerable different Hindu castes. They were all good wage earners, and, as the man, the woman and the children all work, there is no danger of want on account of shortage of money, taking the coolies as a whole. Inquiries regarding diet elicited very vague replies; rice is of course the staple diet but most take meat occasionally, once or twice a week. Little milk is taken. It was very difficult to gauge the amount of vegetables and fat consumed. There is certainly reason to believe that the fat consumption is very low. Some fruit is usually taken and nearly all drink an alcoholic country beer which is reputed to be rich in vitamin-B complex.

*Age and sex.*—There were 26 males and 32 females. The ages varied from 13 to 67 in the first series; grouped by decades they are distributed as follows:—

Decade.	Males.	Females.
10-20 ..	5	11
20-30 ..	8	16
30-40 ..	9	2
40-50 ..	4	2
Over 50 ..	..	1
TOTALS ..	26	32

It will be seen that there is a marked contrast in the sex distribution below and above 30. The difference is statistically very 'significant'.

The age given by coolie patients is, however, always approximate. The same age-sex grouping is not apparent in the second series, but this may be because a number of women with young children were deliberately excluded.

*Technique.*—The method adopted in the case of the normal persons was again followed. Five c.c. of blood was taken from a vein and placed in a small flask containing 0.01 gramme potassium oxalate as an anti-coagulant, and all examinations except the coagulation time and the reticulocyte counts were made from this specimen.

*Hæmoglobin estimation.*—A standardized Hellige hæmometer was used and the readings were taken after 20 minutes, as in our earlier work. The results were recorded as grammes per 100 c.c. of blood.

There were originally 35 women and 26 men selected and examined, but, of these, three women have been excluded from this analysis on the grounds that their hæmoglobin was found, when estimated by the more accurate method, to be well within the 'normal' range, being 8.525 g., 9.35 g., and 9.90 g., respectively. If we adopted the rule that the normal range is the mean  $\pm$  twice the standard deviation, we should have to exclude four others, amongst them one with a hæmoglobin value of 6.87 g.: this would be absurd. We cannot adopt the view that the figure, 10.03 g., which we found to be the mean of 17 so-called 'normal' women coolies, represents the mean value of the hæmoglobin of healthy females of the same race.

The lowest figure was 2.475 g., the highest 7.95 g., and the mean 4.80 g. The cases are grouped according to the hæmoglobin content of their blood at the first examination, as follows:—

Hæmoglobin in grammes per 100 c.c.	Males.	Females.	TOTALS.
2.0—2.99 ..	2	5	7
3.0—3.99 ..	4	7	11
4.0—4.99 ..	6	7	13
5.0—5.99 ..	6	7	13
6.0—6.99 ..	7	3	10
7.0—7.99 ..	1	3	4
TOTALS ..	26	32	58

*Mean corpuscular values.*—We made a cell-volume estimation in a number of cases, but, as we had not standardized our method and had not adopted it as a routine examination at that time, we are not including the cell-volume figures of the first series in this report.

The red cell count was, of course, done so that the mean corpuscular hæmoglobin (MCH) could be worked out for each case.

In Chart 1, the MCH in micro-micro-grammes and the hæmoglobin in grammes per 100 c.c. of blood in these 58 cases are shown. The two lines indicate the normal MCH of, respectively, ( $m^1$ ) the normal city-dwelling male Indians (from Table VI, Part V) and ( $m^2$ ) the 'normal' tea-garden coolies, male and female (worked out from the figures used in Part III).

This chart shows (i) that there is in this series no obvious correlation between the degree of anæmia and the amount of hæmoglobin in each cell, (ii) that, even judged on the low standard of the coolie population ( $m^2 = 22.06 \pm 2.67 \gamma\gamma$ ), nearly three-quarters of the cases are hypochromic, and (iii) that judged on normal Indian standards ( $m^1 = 28.53 \pm 2.31 \gamma\gamma$ ) only two can be said to be hyperchromic.

If we take the line at 20  $\gamma\gamma$  as an arbitrary division, it will be seen that there is a marked concentration of cases below this line and that beyond it they are scattered.

This provides an opportunity to separate the cases into two groups—definitely hypochromic and orthochromic, the latter including two cases that come just beyond the normal limits and therefore might be considered as hyperchromic. We will consider the rest of the data, as a whole and in each of our arbitrary and provisional groups, to see if we can justify this division.

*Sex.*—In the hypochromic series there are 22 males and 20 females: in the orthochromic series there are 4 males and 12 females. There is thus an apparent sex difference in the two series; this difference is nearly but not quite 'significant'\*.

Another way of expressing this difference is by the means of the MCH in the two series; in the males it is 18.5  $\gamma\gamma$  and in the females 19.9  $\gamma\gamma$ . This sex difference in the MCH's does not exist in any non-anæmia series we have so far examined.

*Diameter of the red cells.*—In seven cases selected at random the diameter of 500 cells was measured and the mean and coefficient of variation calculated. These are shown in the table below, arranged according to their size; the MCH in each case is entered to show the correlation between the mean diameters and hæmoglobin

---

\* Wherever the word 'significant' is used, statistical significance of a 0.05 order is implied. To take the case in point, if it could be shown that in a chance distribution a certain degree of female predominance would only occur once in twenty times, that predominance is said to be 'significant'; in this case the chances are a little less than 20 to 1. In this case the calculation is made by the formula:

Standard deviation =  $p^o q^o \left( \frac{1}{n} + \frac{1}{n'} \right)$ . The difference in the percentage of males in the two series is just less than twice the standard deviation. Had the difference been 2 per cent higher it would have been just twice the standard deviation of the difference and would have therefore been considered to be 'significant'.

content of the cells; this is fairly high ( $r = 0.6996$ ). Two sample curves are shown in Charts 1 and 2.

Serial number.	Mean diameter.	Coefficient of variation.	MCH.
43	5.73 $\mu$	0.1595	12.22 $\gamma\gamma$
42	5.99 $\mu$	0.1487	15.63 $\gamma\gamma$
32	6.21 $\mu$	0.1375	17.56 $\gamma\gamma$
55	6.91 $\mu$	0.1295	17.50 $\gamma\gamma$
44	6.92 $\mu$	..	19.91 $\gamma\gamma$
33	6.93 $\mu$	0.1141	26.66 $\gamma\gamma$
29	7.13 $\mu$	0.1198	18.72 $\gamma\gamma$

*Reticulocytes.*—These were counted in each case by the method already described (see Part II). The lowest count was 0.7 per cent, the highest 9.6 per cent, and the mean of the whole series was 4.26 per cent, of the 26 males 4.16 per cent, and of the 32 females 4.33 per cent. The mean for the orthochromic series was 4.83 per cent.

The mean reticulocyte count for the normals of the same population was 2.17 per cent. In this series 21 males and 24 females have a reticulocyte percentage above this mean, the remaining 5 and 8, respectively, being below it.

*Platelets.*—These were counted by the method already described, in all except two cases. The mean of the series of 56 cases was 210,750 per c.mm., of the 25 males 254,080 per c.mm., and of the 31 females 175,807 per c.mm.; the highest count was 735,000 and the lowest 25,000 per c.mm. The mean of the orthochromic series was 153,600 per c.mm. Three males and one female only showed a count above 415,000, the 'normal' mean for the same population.

*Abnormal red cells.*—Anisocytosis and polychromasia were more or less constant findings. Nucleated red cells, normoblasts or macronormoblasts were noted in 13 cases of which 3 were in the orthochromic series.

*Halometric readings.*—We attach no importance to these readings (see Part V) but they are reported. The means amongst the males, the females, and the whole series were, respectively, 7.52 $\mu$ , 7.42 $\mu$ , and 7.48 $\mu$ ; in three males and six females the estimations were below the 'normal' mean of 7.05 $\mu$  (see Part III). In the orthochromic series the mean was 7.39 $\mu$ , that is to say, it was less than the mean of the readings in the hypochromic series, which is a very unlikely contingency.

CHART 1.

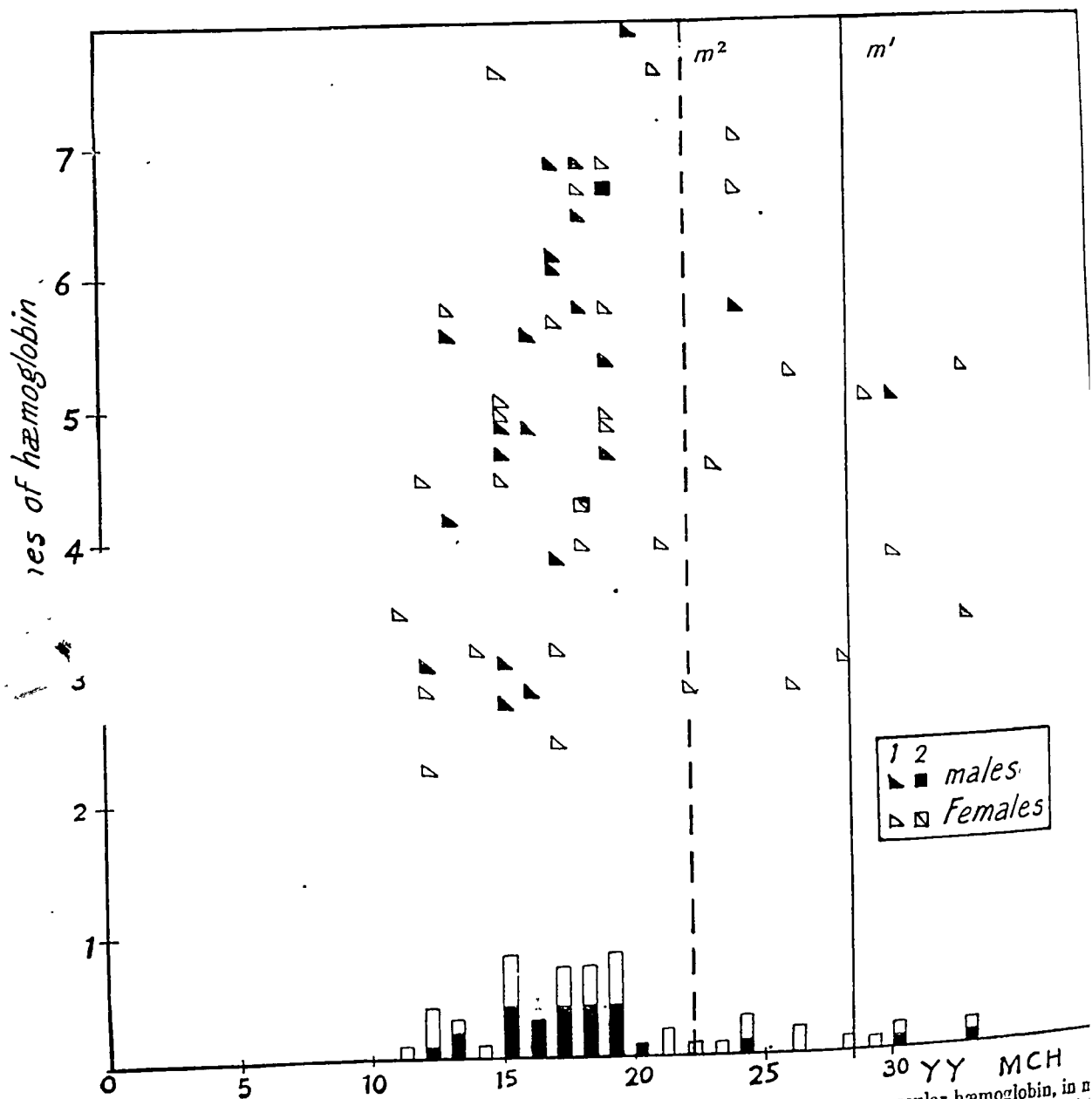


CHART 1. Showing the relationship of the degree of anæmia and the mean corpuscular hæmoglobin, in females;  $m^1$  (line) indicates the normal mean corpuscular hæmoglobin and  $m^2$  (dot line) corpuscular hæmoglobin of the 'normal' coolie population.



CHART 2.

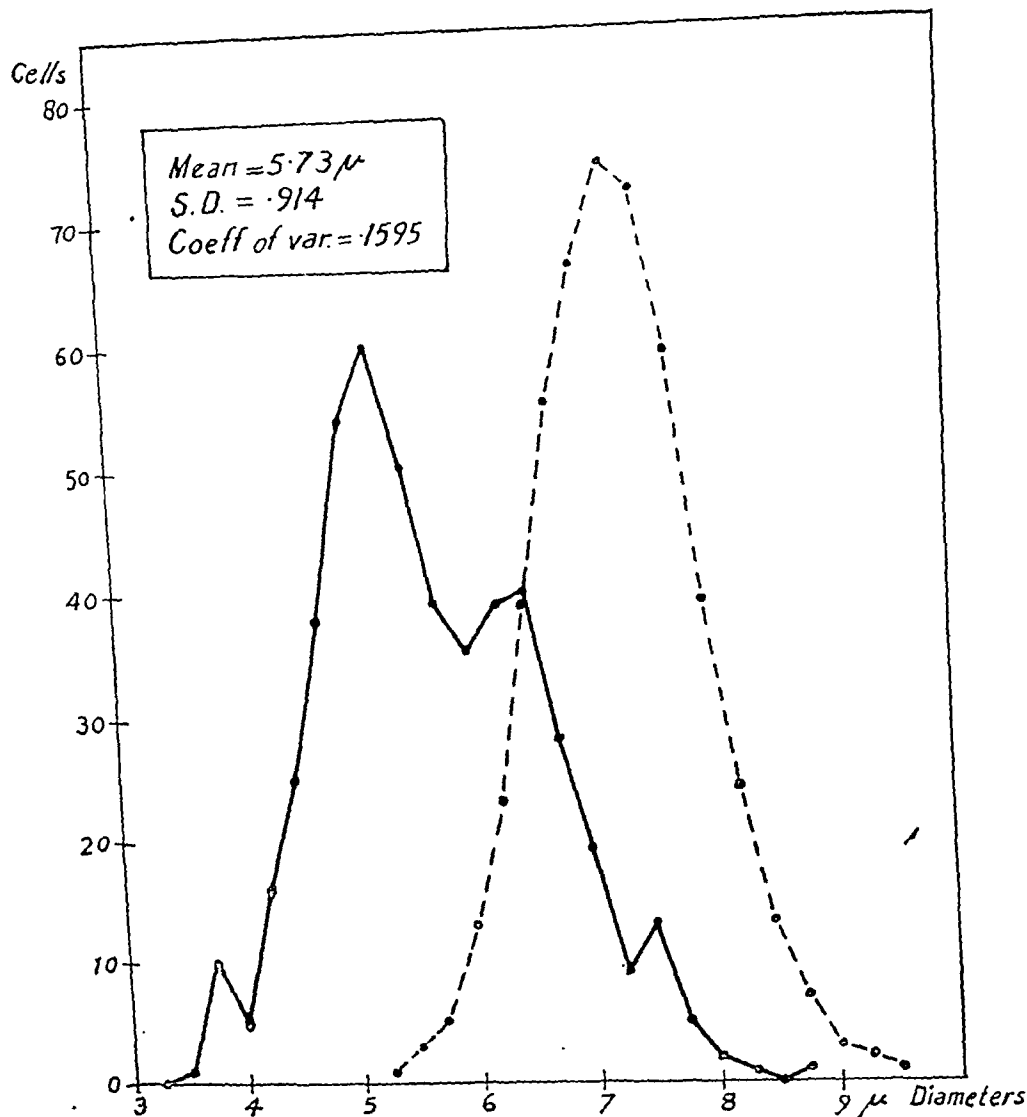


CHART 2. Showing a typical Price-Jones' curve of a hypochromic case; the dot line is a composite curve based on the cell measurements in ten healthy city-dwelling Indians (measured by Dr. P. A. Maplestone).

CHART 3.

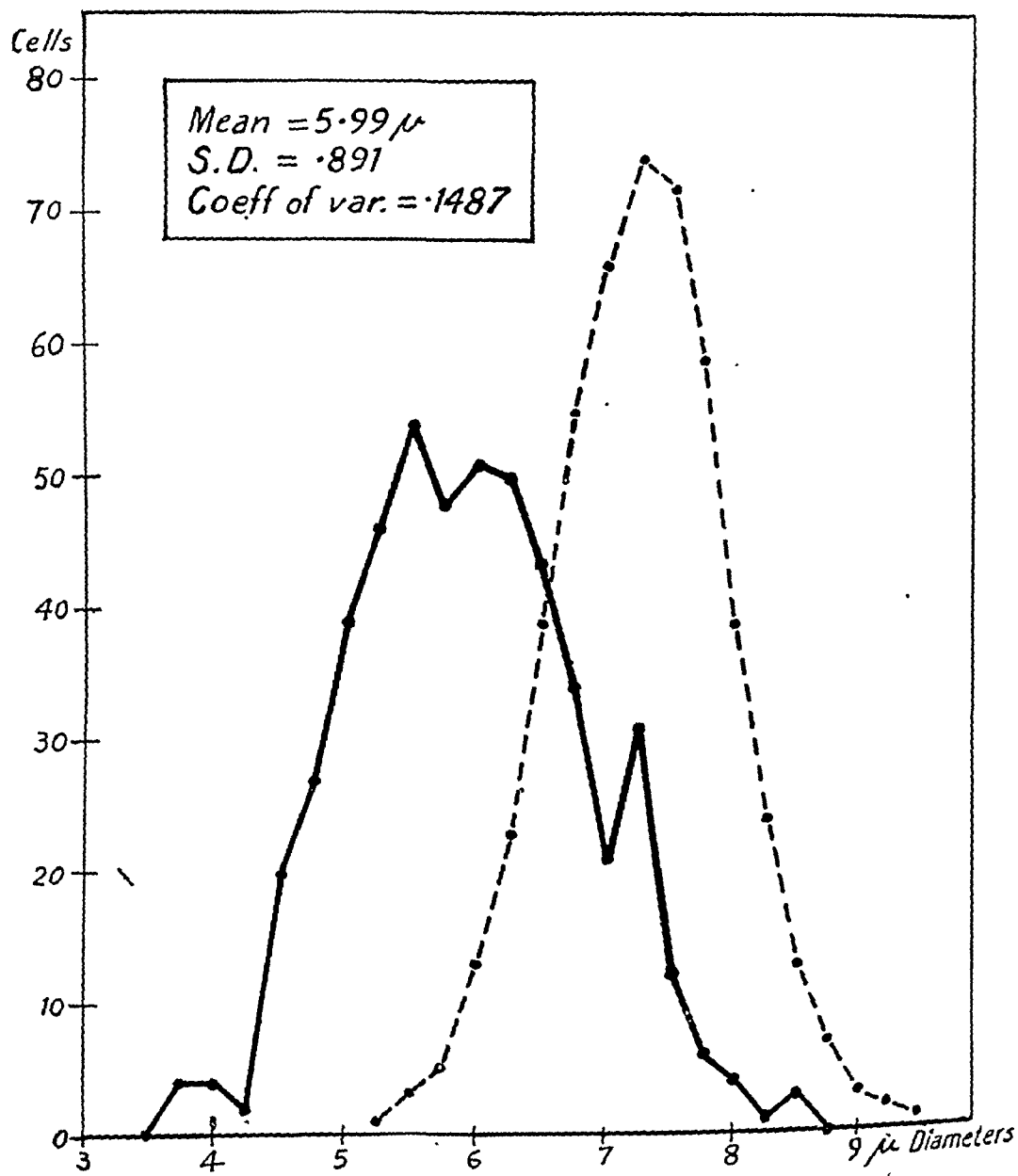


CHART 3. Showing another example of a Price-Jones' curve from a case in this series.



purposes the remainder of the white cells may be considered as having been neutrophil granulocytes.

*The fragility test.*—This was carried out in the usual way with saline solutions of different concentrations; the strength of the strongest solutions (a) in which slight hæmolysis occurred and (b) in which hæmolysis was complete was noted, and the means of these for males, for females, and for the whole series worked out. The figures, based on 21 males and 30 females, are given below :—

	(a)	(b)
	Hæmolysis commenced, per cent.	Hæmolysis complete, per cent.
Males ..	0.3914	0.2319
Females ..	0.4080	0.2350
TOTALS ..	0.4012	0.2337

These figures indicate that there is little change from the normal in the fragility of the red cells; in no individual case was there any marked departure from the normal.

*Coagulation time.*—The method used was to take blood into a capillary tube and break the tube at half-minute intervals. We have not made a habit of keeping the tubes in a water-bath, or incubator, as the temperature in Calcutta is so often about blood temperature. We omitted to do this in Assam where the temperature was colder and varied at different periods of the work; the readings must, therefore, be accepted with reserve.

Only 24 were done, 11 males and 13 females. The means of the coagulation times are given below :—

	Mean of readings (minutes).
Males .. ..	6.27
Females .. ..	6.81
TOTAL .. ..	6.58
Standard deviation of mean ..	6.05

In 35 'normals' the mean was  $4.67 \pm 2.17$  and the difference between males and females was about the same as in this series.

*The van den Bergh tests.*

In no case was the *direct* test positive. We have always encountered great difficulty in obtaining a solution for matching the van den Bergh results; so much so that we have had to give up all attempts to use the colorimeter and have depended on matching the solution approximately in two test-tubes of the same calibre; we have consequently not used units for indicating the results. We have used the sign (+) to indicate bilirubinæmia of from 0.5 mg. to 1 mg. per 100 c.c., + from 1 mg. to 2 mg., and ++ 2 mg. or over.

The results are shown in the table below:—

TABLE I.

Indirect van den Bergh.	Hypochromic.		Orthochromic.		'Normal' coolies.	
	M.	F.	M.	F.	M.	F.
Negative ..	10	5	1	2	10	2
(+) ..	7	8	1	3	3	..
+	4	7	1	4	1	..
++ ..	1	0	..	2	1	..
		30		7		15
		12		7		2

That is to say, only 12 out of 42 of the hypochromic cases but just half of the orthochromic cases gave a positive van den Bergh.

*Van den Bergh and urobilin in the urine.*—Urobilin was not noted as being increased unless the fluorescence was very distinct in a dilution of 1 in 20 of urine. Urobilin was present in abnormal amount in the urine of 15 out of 39 of the hypochromic and in 7 out of 12 of the orthochromic cases. The two tests were done in 47 cases:—

	UROBILIN IN URINE.	
	Definitely increased.	Normal.
Positive van den Bergh ..	8	6
Negative van den Bergh ..	13	20

*Discussion.*—These results are a little surprising for two reasons. There seems to be an unusually high percentage of hyper-bilirubinæmia in the hypochromic cases, in view of the fact that hæmolysis is seldom a feature of this type of anæmia. This led us to carry out the test in a few non-anæmic coolies, as we had unfortunately omitted to do this whilst doing other 'normal' examinations. Circumstances only allowed us to test seventeen 'normal' coolies, of which 15 were men; the results, included in Table I, indicate that there is some imbalance between hæmolysis and utilization of the bilirubin even in these so-called 'normal' people (or there is hepatic dysfunction); if we consider men only the results are not significantly different from those of the hypochromic anæmics.

Secondly, there is a very poor correlation between the van den Bergh and the urobilin in the urine. There is a possible fallacy in our records, as we had to trust patients to bring *their own* urine, whereas we took the blood ourselves. There is little difference between the urobilin findings in the hypochromic and orthochromic cases.

Two points emerge, namely, that there is a difference between the percentage of van den Bergh positive cases in the hypochromic series and in the orthochromic series, and in males and females, respectively, though in neither case is this difference statistically quite significant; there is, therefore, evidence of a greater imbalance between hæmolysis and bilirubin excretion or re-utilization in the orthochromic and amongst female patients. Further, it is suggested by the figures that higher bilirubin content in the females is not dependent upon the fact that more females fall into the orthochromic group as the sex difference is evident in the hypochromic series alone, and inversely, that the higher bilirubin content of the orthochromic series is not dependent on the fact that it consists mainly of females.

The percentage occurrence of hyper-bilirubinæmia in the orthochromic series is significantly greater than in the hypochromic series, and the difference is statistically significant.

*Hyper-bilirubinæmia and splenic enlargement.*—The correlation of the size of the spleen with the van den Bergh reaction was done in 54 cases, and the results are shown below:—

That is, there is hyper-bilirubinæmia in 50 per cent of the cases with an enlarged spleen and in only 25 per cent of those with non-palpable spleens ; this difference is suggestive but not quite significant statistically.

The splenic enlargement was proportionately distributed between the two sexes and between the hypochromic and orthochromic groups, respectively.

*Hookworm infection.*—Almost the whole community is infected. In this series an exact estimation of the hookworm ova was not made but in every case a stool was examined and the intensity of the infection was noted ; the findings were entered as one, two or three *plus*. It is estimated roughly that +++ indicates an infection of 10,000 eggs or more per gramme of stool, ++ between 2,000 and 10,000, + between 200 and 2,000, and — less than 200. The findings in the two groups are as follows :—

Infection.	Hypochromic.	Orthochromic.	Mean of hæmoglobin content of blood in grammes.
+++ ..	12	6	4.33
++ ..	8	4	5.40
+ ..	11	4	5.64
— ..	8	1	4.59
Not examined ..	3	1	..

There is little difference between the two groups but the hookworm infection is slightly higher in the orthochromic group. There seems to be very little sign of correlation between the degree of hookworm infection and the degree of anæmia.

*Eosinophil count and hookworm infection.*—Fifteen cases with a three-*plus* hookworm infection had a mean eosinophil count of 15.1 per cent, whereas eight cases with ova less than 200 per gramme had a mean of 16.75 per cent. There is thus no correlation between the degree of hookworm infection and the eosinophil count.

*Hyper-bilirubinæmia and hookworm infection.*—Two of 18 three-*plus* hookworm-infected cases and 2 out of 8 of those with a few or no ova had a positive van den Bergh reaction, ++ or + ; the proportion is higher in cases with a low hookworm infection, but the difference is not significant.

*Gastric hydrochloric acid.*

Gastric analysis was done in 45 cases.

*Method.*—The technique used was the same as that described in Part IV of this series. A gruel test-meal was given. In no instance was histamine used.

*Achlorhydria.*—There was achlorhydria in 5 cases (8 samples in 2 cases, 7 in one, and 5 in two); all these were females.

Two were from 31 cases of the hypochromic series.

Three were from 14 cases of the orthochromic series.

In one other case, a male in the orthochromic series, the first 7 samples contained no free acid and in the 8th there was a trace.

In two of these six cases a second gastric analysis was done. In the first, a female, the initial hæmoglobin was 2·89 grammes and the mean corpuscular hæmoglobin (MCH) 26·19  $\gamma\gamma$ ; after an interval of 90 days' irregular outpatient treatment the hæmoglobin had only improved to 3·3 g. and the MCH decreased to 21·86  $\gamma\gamma$ , and there was still no free acid. In the second patient, also a female, a second and a third count was done after 74 days and 100 days, respectively. Some distinct improvement in the blood picture had occurred and on both occasions there was some free acid in the later gastric samples:—

Serial number of test.	Hæmoglobin in grammes.	Mean corpuscular hæmoglobin (MCH).	Maximum free acid in any sample.
1st ..	5·09	29·07	0
2nd ..	6·87	19·10	4
3rd ..	8·80	21·67	10

*Fasting stomach.*—Of the remaining 39 cases, from one no gastric juice could be obtained, and from 13 no acid was found in the fasting stomach; these were 5 males and 8 females, three of the latter being in the orthochromic series; the remainder showed varying amounts of free acid, up to a maximum titre of 64 c.c. N/10 NaOH.

*Other samples.*—Of the 40 cases in which some acid was demonstrated, 5 females and 3 males had a maximum free acidity of less than 15 c.c., that is, they showed hypo-acidity; of these 3 (one male) were from amongst the orthochromic cases. Three males and two females showed an acidity of 60 c.c. or more in one or more samples. The highest was 85 c.c. The 3 males were in the hypochromic series and both females were in the orthochromic series.



The mean of the highest observations in each analysis for the 18 males was 36·4 c.c., and for 25 females 25·7 c.c. (one male and one female are omitted as only two samples were taken in each case).

The total acidity was recorded in each case, but as it was always about 10 c.c. higher than the free acidity it is not referred to in this analysis.

The means of all the readings, including the zero readings in the achlorhydric cases, at the different time intervals, for the males, for the females, and for the orthochromic cases, have been calculated and are shown in Table II. They have been plotted on paper and are shown in Charts 4, 5, and 6.

TABLE II.

				Males.	Females.	Orthochromic cases only.
Number examined				19	26	13
Fasting juice				18·8	14·4	9
Time interval						
½ hour	..	..	..	8·5	2·6	1·2
½ "	..	..	..	14·1	6·3	8·0
¾ "	..	..	..	18·7	13·7	15·5
1 "	..	..	..	24·1	16·4	16·3
1½ hours	..	..	..	24·7	21·8	18·0
1½ "	..	..	..	27·4	26·3	23·7
1½ "	..	..	..	22·2	21·4	22·8

CHART 4.

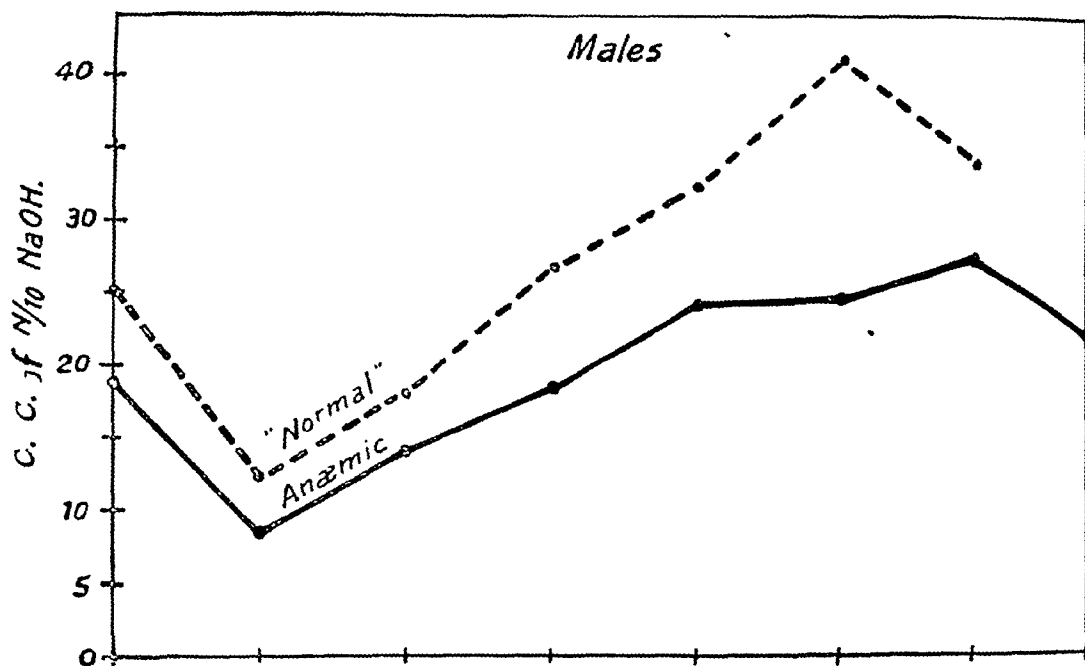
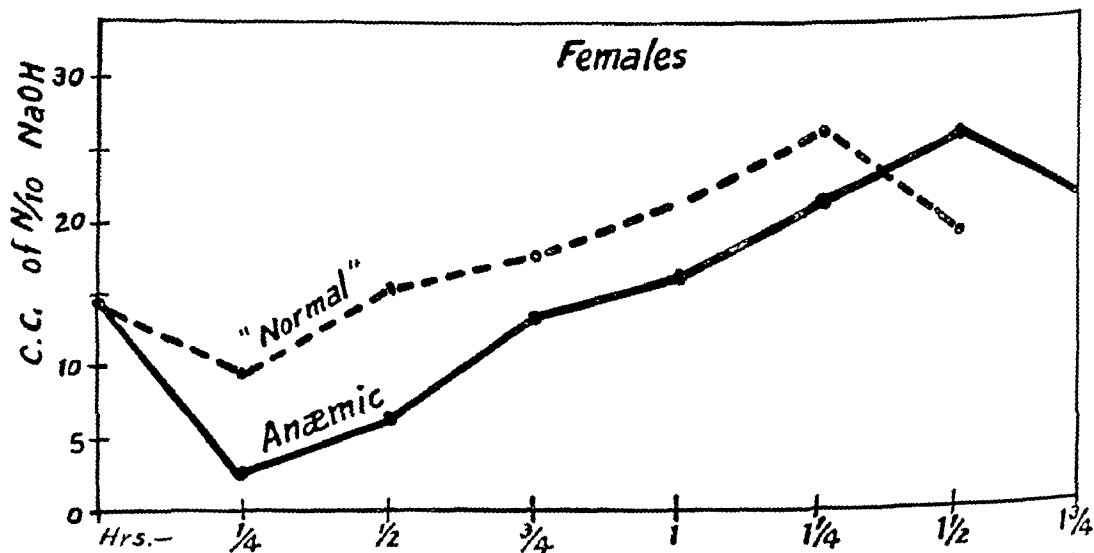


CHART 5.



CHARTS 4 and 5. Showing the mean of the acid curve in cases of anæmia contrasted with the mean curve in 'normal' coolies; the anæmic curves are based on estimations in 19 men and 26 women, respectively. A gruel test-meal was used and no histamine was given.

CHART 6.

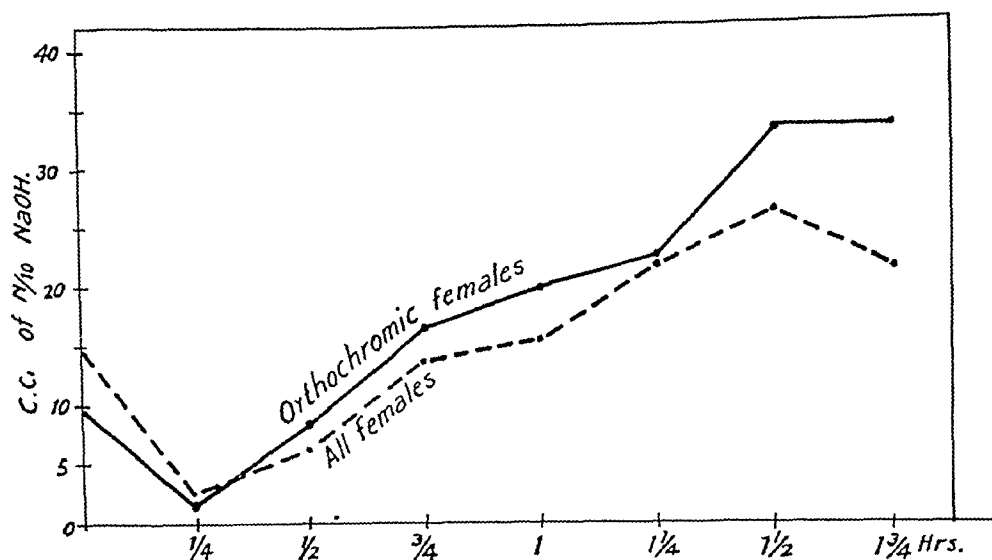


CHART 6. Showing the mean acid curve in 11 females from the orthochromic group and the mean curve in all female anemics; the curves are materially the same.

The gastric analyses are summarized in the following table :—

TABLE III.

	Hypochromic.		Orthochromic.		TOTALS.
	M.	F.	M.	F.	
Total examined ..	16	15	3	11	45
Achlorhydria throughout ..	..	2	..	3	5
No acid in fasting stomach (excluding achlorhydries).	5	5	1	3	14
Hypochlorhydria ..	2	3	1	2	8
Hyperchlorhydria ..	3	..	..	2	5

*Discussion.*—We will first consider the analyses as a whole. The findings do not indicate that the gastric function plays an important part in the ætiology of the anæmia in these cases. It will be seen that the proportion of achlorhydrics is scarcely greater than that in a normal series in Europe or America, when it is remembered that histamine was not given in any of these cases. However, there was only one achlorhydric in 43 'normal' persons of the same community (see Part IV). Though the mean curve is well within the normal limits of the European standard, it is slightly lower than the mean curve of 'normals' of the same population.

The mean curve of the female patients is distinctly lower than that of the males, as was the case in the normal series. Charts 4 and 5 show the curves for normals and anæmics, for men and women, respectively. In the males the curve of the anæmics is distinctly lower than that of the 'normals', but in the case of the females the curves are almost identical, except that in the anæmics it is later in reaching its maximum.

If the hypochromic group is considered separately, it will be seen that only two out of the thirty-one cases were achlorhydric; in five others the gastric secretion was below normal. There seems to be little evidence here that the anæmia could be due to failure of iron absorption through deficiency of free acid; on the other hand, it does seem probable that the slight default in gastric secretion was due to the marked anæmia.

In the orthochromic series the percentage of achlorhydria was higher—3 out of 14 cases. The man in whom there was no free acid in the first 7 samples also belonged to this group. Two other cases were hypochlorhydric, but in the remainder of the cases of this series good quantities of free acid were found, so that the mean curve is actually higher than the mean curve of the whole series at most time intervals, if allowance for sex is made (Chart 6).

Of the three achlorhydric orthochromic cases, one showed some secretion when subsequently examined, and in another there was a marked reduction in the MCH at the time of the second examination; the third case, with an MCH of 21.4  $\gamma\gamma$ , would have been classified as hypochromic on European standards. Therefore none of these could be considered as a case of the pernicious anæmia type.

### *Results of treatment.*

For this part of the Inquiry we decided that we ourselves would not undertake any treatment of these patients, nor in any way interfere with the usual procedure on the gardens in which the cases were found. However, it was the routine practice to examine every coolie on the estates at this time of year, and to give to all the anæmic patients a course of treatment for hookworm infection, followed by an iron tonic or other treatment that seemed to be indicated. Every patient in this series had one or more doses of oil of chenopodium and was given iron and ammonium citrate in pharmacopœial doses. In a few instances the patient was admitted to hospital.

*Subsequent examination.*—One or more subsequent examinations were made in 33 cases—12 men and 21 women; the interval between the first and last examinations varied from 45 to 120 days and averaged about 75 days. Of these only seven patients improved appreciably, that is to say, their hæmoglobin increased

by 3 grammes or more; in 6 cases there was either no improvement, or an actual decrease in hæmoglobin. The cases can be divided into the following groups according to the degree of improvement that they showed:—

TABLE IV.

*Showing the results of treatment: 1st series.*

Hæmoglobin increase in grammes per 100 c.c. of blood.	Hypochromic.		Orthochromic.		TOTALS.	Hospital treated.
	M.	F.	M.	F.		
3.0 or more .. ..	2	4	..	1	7	4
2.0 or more, less than 3.0	1	4	..	..	5	1
1.0 „ „ „ „ 2.0	4	3	1	..	8	..
Less than 1.0 .. ..	2	1	..	4	7	1
Decrease .. ..	2	2	.	2	6	1

*Discussion.*—The result of treatment in these cases was very disappointing. The main reason for the failure of response to treatment was that the patients did not attend regularly for their iron-tonic treatment. This is shown by the fact that of the 7 cases in which there were more than 3 grammes improvement 4 were hospital patients, and of the 26 that showed less or no improvement only 3 were hospital patients; the difference is statistically very significant.

The improvement was distinctly better in the hypochromic cases as a whole; the one patient in the orthochromic group who improved was a hospital patient for some time; she had a good diet and possibly (we have no record of this) liver extract, as she was achlorhydric at first; if females only are considered, the difference between the improvement in the hypochromic cases and the orthochromic is more striking.

The change in the MCH between the first and the last counts was somewhat confusing; the MCH of the orthochromic cases decreased in every instance, slightly in 7 and markedly in 1, and in the hypochromic cases it increased in 19 out of 26 cases and in 6 of these sufficiently to bring it above the 20  $\gamma\gamma$  mark.

Most of the other blood examinations were repeated but the findings show so little change that the results are not worth analysing.

#### *Conclusions based on observation made in the first series.*

The only point we need consider now is whether the purely arbitrary division into hypochromic and orthochromic was justified by the other findings.

There is a preponderance of females in the orthochromic group but the difference is not significant statistically. No difference between the two groups was noted in the other blood cell counts, nor in the fragility or coagulation time.

The incidence of hyper-bilirubinæmia was definitely higher in the orthochromic series, but the difference is not statistically significant. Again there were more cases of achlorhydria amongst the orthochromic. The most striking difference was in the response to treatment, and, if females only are considered, the difference in the response between the two groups is statistically significant, but not much importance can be attached to this as the treatment was so irregular.

It, therefore, seems doubtful on the data we have analysed whether they can be considered as two separate groups, though perhaps we are justified in saying that females tend to have more heavily hæmoglobinized (and therefore probably larger) cells, and that those cases with the more heavily hæmoglobinized cells show a greater tendency to hyper-bilirubinæmia and achlorhydria, and do not respond so well to treatment by anthelmintics and iron.

### THE SECOND SERIES.

The results of treatment in the first series were so disappointing that we decided to undertake another series during the following cold weather (1934-1935), to make special arrangements about the treatment of the patients, and to make more frequent examinations during the treatment. We felt that it would be advantageous to augment the series by bringing the number of anæmics examined up to at least a hundred, and to carry out more complete examinations.

In the second series, a certain amount of selection was exercised and a greater degree of anæmia was demanded. The highest hæmoglobin percentage in the series was 6.19 grammes per 100 c.c. of blood. No case was excluded on account of the special nature of his or her anæmia, other than the degree of anæmia, but, as certain dietary experiments were envisaged, notorious non-co-operators and women who had young children, to whom they might be tempted to give the extra food that was provided, were excluded.

The examinations carried out were much on the same lines as in the first series, with the few exceptions enumerated below :—

- (a) The cell volume was estimated in every case, so that the mean corpuscular volume and the mean corpuscular hæmoglobin concentration, as well as the mean corpuscular hæmoglobin could be worked out.
- (b) The hæmoglobin and the cell-volume estimations, and the red-cell and reticulocyte counts were done at frequent intervals during the course of the treatment and during 'convalescence', and in addition the white-cell count, the van den Bergh reaction, and certain other examinations were done at the end of the investigation.
- (c) A more accurate quantitative estimation of the hookworm infection was made.
- (d) The weight of the patient was taken at the beginning and the end of the investigation.
- (e) Fractional gastric analysis was attempted in every case; the first eight patients refused to swallow the gruel or vomited out the tube immediately they had swallowed it. (In this respect the average tea-garden coolie is very sheep-like; all these patients were on one garden and all the tests were done at the same time; if the first patient had kept the tube down they would probably all have kept theirs down). After this we used the alcohol 'test-meal'; this was much more popular and no difficulties arose. A fractional meal was done at a later date in the first eight cases.
- (f) For the coagulation-time test the blood was kept in warm water (37°C.).
- (g) A platelet count was not done except in a few instances and the fragility test was frequently omitted.
- (h) For the reticulocyte count a few drops of blood were taken into a small tube containing an equal quantity of cresyl blue, 1 per cent in normal saline, and a count was made on a 'wet' preparation.

*The clinical material.*—This was from exactly the same source as in the first series; the patients were tea-garden coolies from 5 different gardens; all but 6 were hospital patients. They presented very much the same clinical picture as those in the first series.

*Age and sex.*—There were originally 18 men and 24 women in the experiment; one female absconded after the first count (hæmoglobin = 2.95 g., MCH = 12.5  $\gamma\gamma$ , MCV = 52.8 cu.  $\mu$ , MCHC = 23.6 per cent, and a normal gastric acidity) and has been excluded from further consideration in this series.

The remainder fall into the following age-sex groups:—

Decade.	Male.	Female.
10—20 ..	6	7
20—30 ..	6	7
30—40 ..	3	7
40—50 ..	3	2

*Hæmoglobin.*—According to the hæmoglobin content of their blood the cases can be grouped as follows:—

Ranges in g. per 100 c.c.	Males.	Females.	TOTALS.
2.00 to 2.99 ..	2	3	5
3.00 to 3.99 ..	6	5	11
4.00 to 4.99 ..	5	8	13
5.00 to 5.99 ..	4	6	10
6.00 to 6.99 ..	1	1	2

The highest was 6.19 g., the lowest 2.40 g., and the mean of the series 4.31 g.

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*The red-cell count.*—According to their initial red-cell count the cases can be grouped as follows:—

Red-cell count in millions per c.mm.	Males.	Females.	TOTALS.
1.00 to 1.49 ..	1	1	2
1.50 to 1.99 ..	3	4	7
2.00 to 2.49 ..	5	7	12
2.50 to 2.99 ..	3	4	7
3.00 to 3.49 ..	4	6	10
4.00 to 4.49 ..	2	1	3

*The mean corpuscular values.*

The mean corpuscular hæmoglobin was calculated in all cases, and the mean corpuscular volume and the mean corpuscular hæmoglobin concentration in 38 cases: these are shown below:—

	Males.	Females.	All cases.
Mean corpuscular hæmoglobin (MCH) ..	16.17 $\gamma\gamma$	18.06 $\gamma\gamma$	17.23 $\gamma\gamma$
„ „ volume (MCV) ..	65.75 cu. $\mu$	72.60 cu. $\mu$	69.72 cu. $\mu$
„ „ hæmoglobin concentration (MCHC)	25.62 per cent.	25.60 per cent.	25.61 per cent.

Thus, compared with the first series, the MCH is slightly lower, and, as before, it is higher in the females than in males. The standard error of the mean for the males is  $\pm 0.606$  and for the females  $\pm 0.681$  and the standard error of the difference is  $\pm 0.912$ ; the difference between the two means, which is 1.89  $\gamma\gamma$ , is therefore just significant. All the cases but 2 are definitely hypochromic; even these 2 are below the normal MCH of 29  $\gamma\gamma$ .

On the other hand, although all but 8 cases would be considered microcytic on ordinary standards, the mean of the series is not much lower than the mean of the so-called normal coolies.



The mean size of the cells in the male series is distinctly smaller than of those in the female series, but as the dispersion is considerable the difference between the means is not quite significant.

In Chart 7 the relationship of the corpuscular hæmoglobin to the corpuscular volume in 38 cases (in 3 the MCV was not recorded) is shown. This shows a fairly

CHART 7.

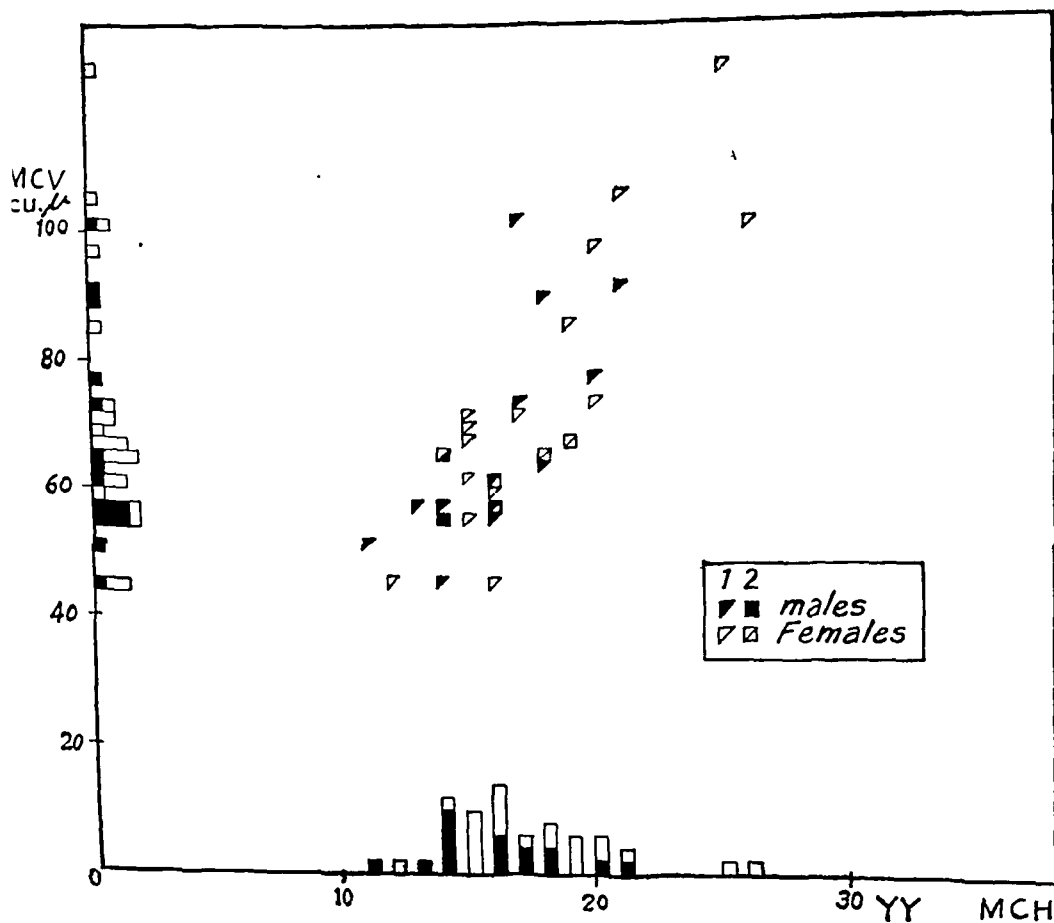


CHART 7. Showing the relationship between the mean corpuscular volume and the mean corpuscular hæmoglobin in 38 cases in the second series.

high coefficient of correlation, but it is apparent from the obtuseness of the angle which the mean line (not drawn) of these plottings makes with the base line that the mean corpuscular hæmoglobin concentration is low.

*The reticulocyte count, and the total and differential white-cell counts.*—These counts were done in every case; the results do not differ materially from those in the first series; they are given in tabular form below:—

TABLE V.

	Maximum.	Minimum.	Mean male.	Mean female.	Mean of series.
Reticulocyte, per cent ..	10·4	0·07	3·70	4·10	3·92
Leucocytes, in thousands per c.mm.	10·0	1·875	5·53	5·56	5·55
Lymphocytes, per cent ..	54	14	27·05	33·23	30·45
Large mononuclears, per cent.	17	1	6·61	7·19	6·92
Eosinophils, per cent ..	47	2	13·61	16·55	15·22

Few abnormal white cells were recorded in these counts so that the balance may be considered to be neutrophil polymorphonuclears; the mean of these was just under 50 per cent. In the first series the lymphocytes were about 5 per cent fewer and the polymorphonuclears correspondingly higher.

Nucleated red cells were noted in 5 cases only.

*The van den Bergh reaction.*

In no case was the direct van den Bergh positive.

The indirect test was done in every instance before and after treatment; the results are shown in the following table:—

TABLE VI.

Indirect van den Bergh.	BEFORE TREATMENT.		AFTER TREATMENT.			
	Males.	Females.	++	+	(+)	Negative.
Negative ..	7	10 } 26 {	..	1	..	16
(+) ..	5	4 } {	1	1	..	7
+ ..	5	6 } 15 {	..	2	1	8
++ ..	1	3 } {	1	1	..	2
			7		34	

Of the 15 van den Bergh-positive cases, 5, that is, 33 per cent, had a hookworm egg-count over 10,000; of the van den Bergh-negative cases 57 per cent

had this degree of hookworm infection. There is thus a tendency towards a negative correlation, as in the first series, but the difference is again not significant.

The association of hyper-bilirubinæmia with a relatively high MCH noticed in the first series is not apparent in this series; the mean of the MCH of the van den Bergh-positive cases is 16.84  $\gamma\gamma$  against a mean of 17.23  $\gamma\gamma$  for the whole series.

#### *Urobilin in the urine.*

The urine was tested for urobilin before treatment in 39 cases:—

	Urobilin in excess.	Normal.
Positive van den Bergh cases ..	8	5
Negative „ „ „ „ ..	5	21

*Splenic enlargement.*—The spleen was palpably enlarged in 13 out of 41 cases. The correlation between hyper-bilirubinæmia and enlarged spleen that was noticed in the first series is not apparent here. Of the 15 cases with a positive van den Bergh only 4 had an enlarged spleen.

#### *Fractional gastric analyses.*

The same technique was adopted except that 50 c.c. of 7 per cent alcohol was given as the 'test-meal'.

*Achlorhydria.*—Of the 33 cases examined before treatment, in which at least 8 samples were taken, there was only one case of achlorhydria (hæmoglobin percentage = 3.99 g.; MCH = 19.9); this was a female. In this case a second test was done and histamine given after the fifth acid-free sample; there was 20 c.c. free acid in the seventh sample, 25 c.c. in the eighth, 20 c.c. in the ninth, and 10 c.c. in the tenth.

*Fasting stomach.*—This was tested in all the 41 cases. The amount of juice varied from 5 c.c. to 80 c.c.; the mean was 25 c.c.; in 18 males it was 32 c.c. and in 23 females 20 c.c.

In 5 males and 10 females there was no free acid; in the rest the free acid varied from 4 c.c. to 85 c.c. N/10 HCl.

*Other samples.*—Post-prandial samples were only taken in 33 cases, before treatment.

Of the 32 cases in which free acid was demonstrated 3 had a maximum acidity below 15 c.c. and 3 others between 15 c.c. and 25 c.c. These six cases might be classed as hypochlorhydric in view of the higher average of the acidity in this whole series; 4 were women.

In 15 cases the maximum acidity was 60 c.c. or more; of these 10 were men. If 75 c.c. are taken as the criterion, for hyperacidity, there were 7 cases of which 5 were males with this or a higher degree of acidity.

In nine of the 16 women with free acid, the maximum acidity was reached at the third (5 cases) or fourth post-prandial sample. In 10 of the 16 males the maximum was reached at the second (7 cases) or third post-prandial sample.

The means of the samples at different time intervals are shown in Chart 8 and in the table below:—

CHART 8.

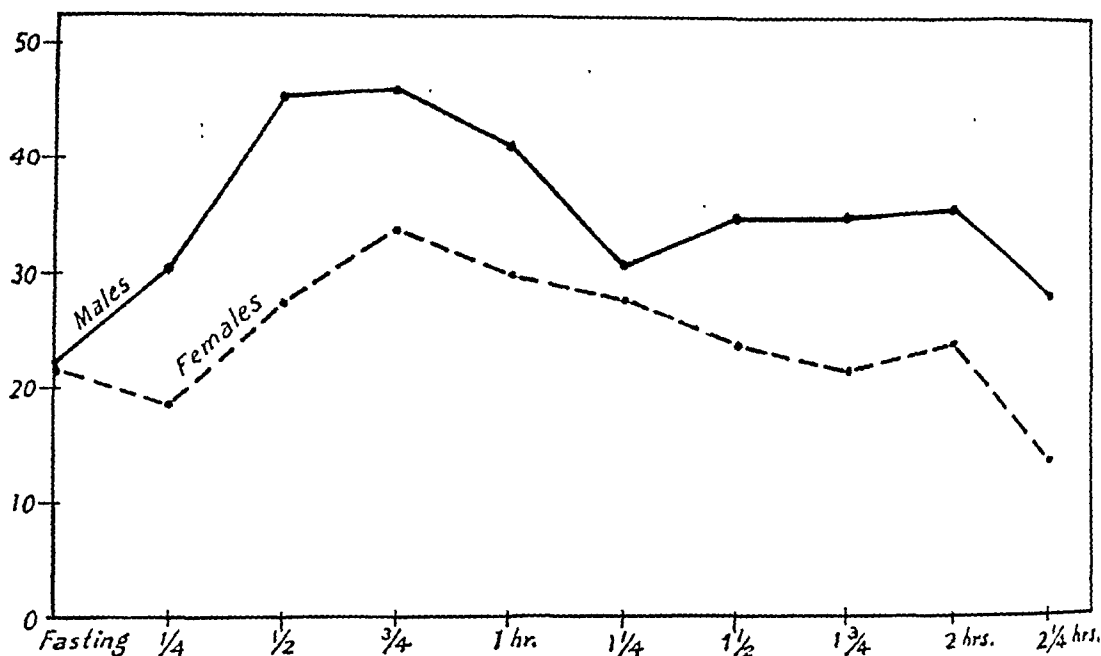


CHART 8. Showing the mean acid curve in males and females of the second series. The alcohol 'test-meal' was used; no histamine was given.

The mean of the highest observation in each test amongst the males was 57.27 c.c. and amongst the females 43.18 c.c. (the achlorhydric case included).

TABLE VII.

		Males.	Females.
Time interval	Fasting juice	22.33	21.74
1/4 hour	..	30.33	18.82
1/2 "	..	45.64	27.88
3/4 "	..	46.28	34.12
1 "	..	41.14	30.12
1 1/4 hours	..	30.87	28.25
1 1/2 "	..	35.00	23.94
1 3/4 "	..	35.00	21.43
2 "	..	35.91 (11 cases)	23.83 (12 cases)
2 1/4 "	..	23.00 ( 5 "	13.67 ( 6 "
2 1/2 "	..	55.00 ( 2 "	12.25 ( 4 "

*Re-examination after treatment.*—This was not undertaken systematically. The re-examination in the achlorhydric case referred to above was after treatment. The 8 cases in which only an examination of the fasting juice was done were re-examined after treatment. One, a male patient, was achlorhydric in 5 samples, after which no juice could be obtained, 3 other cases were hypochlorhydric, and 4 had acidity within the normal range.

*Discussion.*—From the results that we obtained it is quite obvious that the alcohol meal stimulates gastric secretion much more than the gruel meal. The initial fall is absent in the majority of cases and the average readings are from 10 c.c. to 20 c.c. higher than in the gruel series.

In both the males and the females of the second series the mean acidity of the fasting stomach happened to be higher also; the difference is slight in the male cases but more marked in the female cases. Amongst the female cases there were actually more with complete absence of acid in the fasting stomach in the second series, but there were 4 cases with very high acid contents, 2 with a titre at 85 c.c. and 2 at 65 c.c., so that the mean is raised.

What is even more apparent than the height of the acidity is the earlier achievement of the maximum; this was delayed to about  $1\frac{1}{2}$  hours in the first series, whereas amongst the male cases in the second series a number reached their maximum in half an hour, and the mean maximum was at  $\frac{3}{4}$  hour in both male and female. Again, the acid curve rises earlier in the male than in the female cases, but in this series it also rises much higher.

There is evidence of a secondary rise in the curves at two hours; this was not apparent in the first series as the tests were not continued long enough.

It is unfortunate that we have no series of 'normal' observations in Indians in which this alcohol meal has been used.

Except for these few differences in the gastric analyses, which are almost certainly due to the difference in the meal, the findings support our main conclusion based on the first series, namely, that deficiency in acid secretion can play no part on the ætiology of the anæmia in these cases.

### *Hookworm infection.*

The stools were examined in every case for helminthic ova at the beginning of the experiment and again after treatment had been given. In most cases intermediate examinations were done but we are not recording these.

*Technique.*—The egg-counts were made by placing a given bulk (3 g.) of stool, measured by fluid displacement, in a test-tube, making up to 90 c.c. with N/10 caustic soda, transferring the whole to a bottle, and mixing thoroughly with the help of glass beads; 0.15 c.c. of diluted stool is placed on a glass slide, covered with a large coverslip, and all the ova on the slide counted. The dilution is arranged so that the number of eggs on the slide multiplied by 200 gives the total number of eggs per gramme of stool.

All methods of egg-counting are essentially rough, and give little accurate information as to the load of infection in an individual case, but this method probably gives a fairly accurate indication of the degree of infection in a group of cases.

Hookworm, ascaris and whip-worm ova were found in practically every case. The ascaris infection was often very high, but as there is little reason to associate this infection with anæmia we shall only consider the hookworm infection here.

The results of the initial hookworm egg-counts are given below:—

Load. Eggs per gramme.	Number of cases.
(a) Over 10,000 .. ..	21
(b) Less than 10,000 but over 5,000 ..	10
(c) Less than 5,000 but over 2,000 ..	6
(d) Less than 2,000 but 200 or over ..	4
(e) Less than 200 .. ..	0

These figures suggest that the infection was rather heavier in this than in the first series, but it must be remembered that in all the cases of the first series accurate counts were not made.

These egg-counts indicate that the hookworm infection rate amongst the anæmic coolies is very high, in fact 100 per cent. They also show that the load of worms is considerable in the majority. As in the first series, there is no correlation between the degree of anæmia and the degree of the hookworm infection. The 21 cases in which there are 10,000 or more eggs per gramme of stool are distributed as follows:—

TABLE VIII.

Initial hæmoglobin in g. per 100 c.c.	Distribution of cases.	Distribution of heavily infected cases, 10,000 eggs or over.
Less than 3 g. ..	5	2
Over 3 g. but less than 4 g. ..	11	7
„ 4 g. „ „ „ 5 g. ..	13	6
„ 5 g. „ „ „ 6 g. ..	10	6
„ 6 g. .. ..	2	..

*Control counts.*—We counted the eggs in over 500 random samples of stools from the general coolie population on the same gardens, but the figures had to be discarded as the technique adopted was open to criticism. From stool examination in a smaller number of 'normal' coolies (42) we found that the incidence of infection in the general coolie population was almost 100 per cent, but that only about 14 per cent had counts above 10,000, against 51 per cent in the present series.

The higher incidence of heavy infections amongst the anæmic coolies does suggest that hookworm infection plays some part in the ætiology of this anæmia, though the absence of any relationship between the degree of the anæmia and the number of ova in the stools does not support this suggestion.

*Coagulation time.*—This was done in only 25 cases in the second series. The mean coagulation time was 2.88 seconds; it was about the same in males and females. The marked difference in these results and those of the first series is due to a difference in technique. It is evident that there is no marked departure from the normal.

*The results of treatment.*

In the second series of cases we arranged with the doctors in charge of the cases to undertake the anti-anæmic treatment ourselves. The course of treatment

CHART 9.

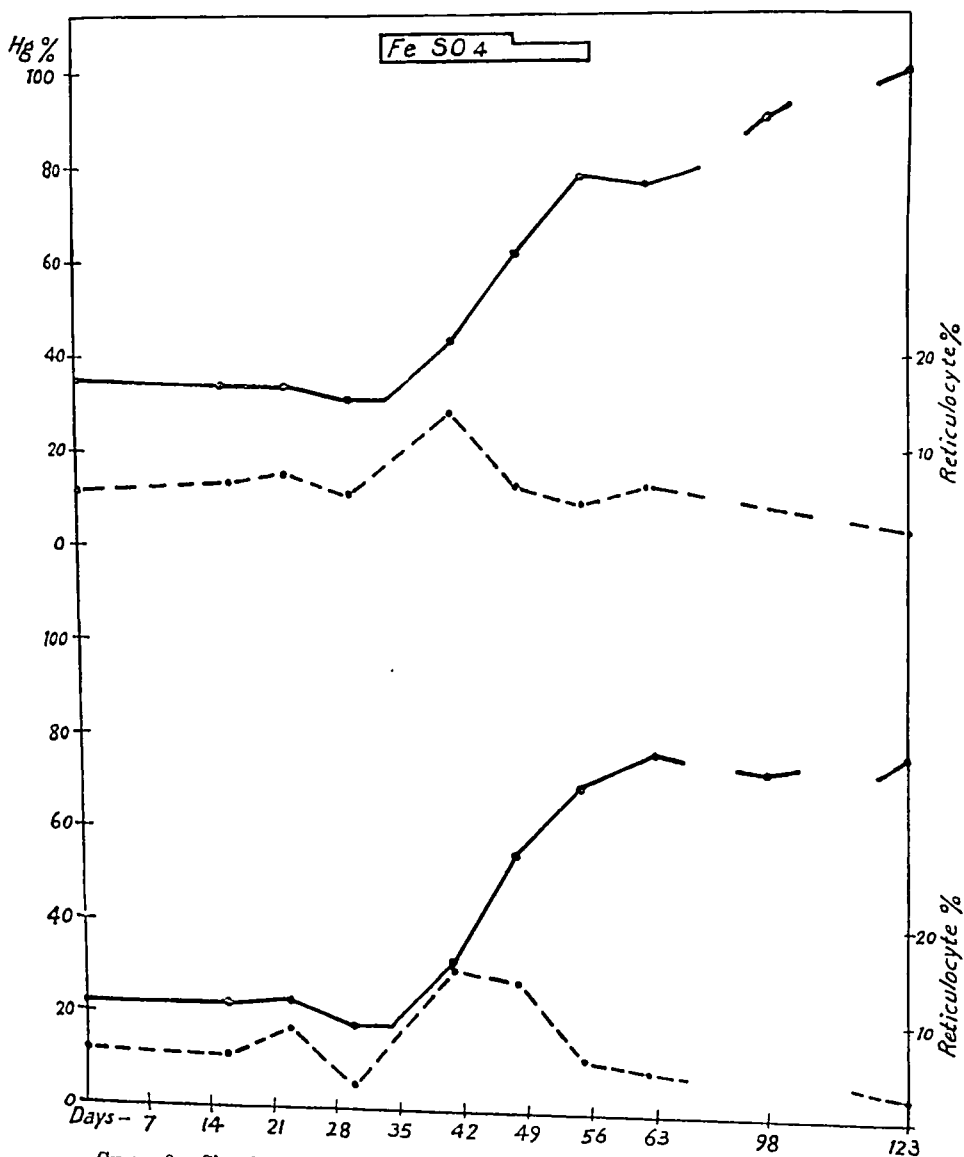


CHART 9. Showing the response to iron therapy in 2 patients who were kept on a good diet of high caloric value for one month before and during treatment. Anthelmintic treatment was given after the iron administration had been discontinued. (— = hæmoglobin; --- = reticulocytes).

and the mode of procedure were varied from garden to garden ; the different results obtained will be discussed in detail below. In every case the 'specific' treatment

CHART 10.

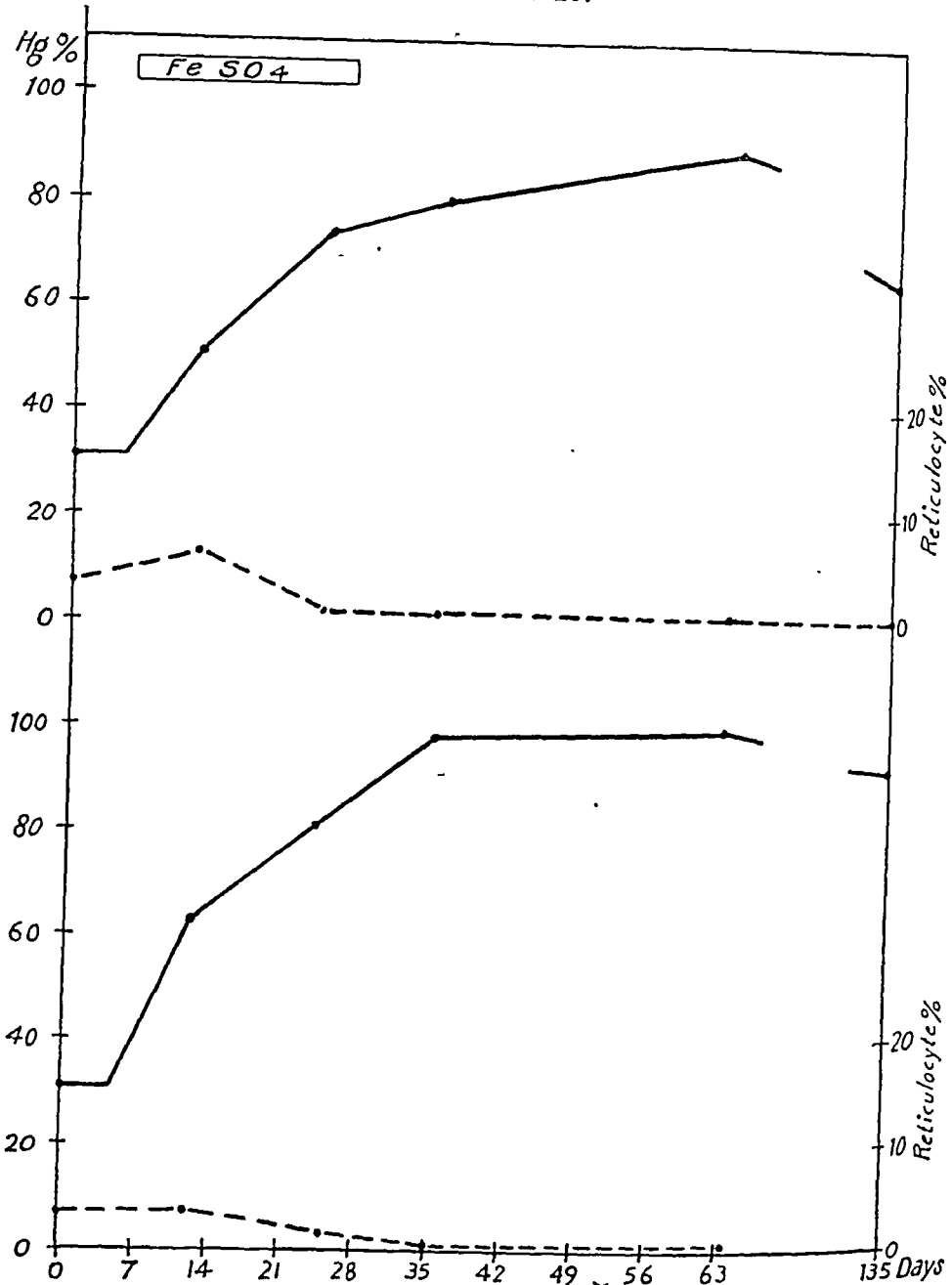


CHART 10. Showing the response to iron therapy in patients who were on a normal diet throughout. Anthelmintic treatment was given after the iron treatment. (— = hæmoglobin ; - - - = reticulocytes).

given was ferrous sulphate. We used the ferrous sulphate in two forms, Glaxo tablets which contain 3 grains of the iron salt, and a trace of copper and



manganese, and tablets made for us by Messrs. Smith, Stanistreet and Co.; these latter contained  $5\frac{1}{2}$  grains of the iron salt, also with a trace of copper and manganese. From considerable experience with these two preparations we have every reason to believe that the ferrous sulphate contained in either of these tablets is fully active. All but 6 patients were in hospital throughout the course of treatment.

We will first consider the results of treatment as a whole.

Thirty-nine out of 41 patients responded to treatment in varying degrees; the poorest response in these cases was in a patient in whom the hæmoglobin increased from 3.07 g. to 4.40 g., that is by 1.33 g. (or 9.5 per cent), and the best in a patient in whom the improvement was from 3.99 g. to 15.81 g., that is 11.82 g. (or 86 per cent). The period of observation was about four months in all but one group of 7 cases in which it was three months, and the improvement referred to here is the difference between the first and the final estimations of hæmoglobin. The characteristic improvement following iron treatment in individual cases is shown in Charts 9 and 10.

According to the degree of improvement the cases can be grouped as follows:—

TABLE IX.

*Showing distribution of cases according to degrees of improvement during treatment.*

			Male.	Female.	TOTALS.	Initial egg- count over 10,000.	Final egg- count over 5,000.	Van den Bergh- positive cases.
Decrease of less than 1 g.	..	..	..	1	1	1	1	..
Improved less than 1 g.	..	..	..	1	1	1	1	..
Improved 1 g. but less than 2 g.	..	..	1	..	1	1	..	..
" 2 " " " " 3 "	..	..	2	..	2	2	2	1
" 3 " " " " 4 "	..	..	1	..	1	1	1	..
" 4 " " " " 5 "	..	..	2	1	3	..	..	..
" 5 " " " " 6 "	..	..	3	4	7	5	2	1
" 6 " " " " 7 "	..	..	1	5	6	3	..	3
" 7 " " " " 8 "	..	..	2	6	8	2	1	6
" 8 " " " " 9 "	..	..	1	5	7	3	4	1
" 9 " " " " 10 "	..	..	4	..	3	1	1	2
" 10 " " " " 11 "	..	..	..	..	..	..	..	..
" 11 " " " " 12 "	..	..	1	..	1	1	..	1
TOTALS ..			18	23	41	21	13	15

Calculated from this table, the mean improvement in the whole series is 6.38 g., in the males 6.39 g., and in the females 6.37 g. The four best results

obtained were in males and the two cases that failed to respond were females, but on the whole the response to treatment in the two sexes was equal.

According to the final level of hæmoglobin reached the cases can be grouped as follows :—

TABLE X.

*Showing distribution of cases according to final hæmoglobin level.*

			Males.	Females.	TOTALS.
15 g. but less than 16 g.	..	..	1	..	1
14 „ „ „ „ 15 „	..	..	2	..	2
13 „ „ „ „ 14 „	..	..	3	2	5
12 „ „ „ „ 13 „	..	..	3	4	7
11 „ „ „ „ 12 „	..	..	..	7	7
10 „ „ „ „ 11 „	..	..	1	3	4
9 „ „ „ „ 10 „	..	..	..	4	4
8 „ „ „ „ 9 „	..	..	4	1	5
7 „ „ „ „ 8 „	..	..	2†	..	2
6 „ „ „ „ 7 „	..	..	1	..	1
4 „ „ „ „ 5 „	..	..	1*	2*	3

*Note.*—For reasons given later in the paper, four cases have been classed as ‘unsatisfactory’.

\* Cases classed as ‘unsatisfactory’.

† One of these was ‘unsatisfactory’.

If the 4 ‘unsatisfactory’ cases are excluded, the mean of the whole series, calculated from the above table, is 11·26 g., of the males 11·32 g. and of the females 11·21 g.

In the original hæmoglobin estimations in this series the tendency of males to have a higher hæmoglobin percentage is shown only in the greater number of females in the series. In the above table it will be seen that the male cases fall into two groups: 12 grammes and over, and 8 grammes and under; 2 of the lower group are the cases referred to as ‘unsatisfactory’, and 5 out of 6 of the remainder come from the garden (*Kharikatea*, *v.i.*) on which the results of treatment on the whole were poor. The inclusion of this group, in which there is reason to suppose that the effect of treatment was not maximal through extraneous causes, in the male series has marked the re-establishment of male superiority in hæmoglobin percentage. The mean in the female series, excluding the two ‘unsatisfactory’ cases, is more than a gramme higher than the mean of ‘normal’ coolie female population (*vide* Part III).

The improvement in relationship to the initial hæmoglobin level is shown in the table below :—

TABLE XI.

Group.	Number in group.	Mean of initial hæmoglobin.	Mean of final hæmoglobin.	Difference.
2.00 to 2.99 .. ..	5	2.64	9.27	6.63
3.00 to 3.99 .. ..	11 (9)	3.41 (3.47)	9.95 (10.84)	6.54 (7.37)
4.00 to 4.99 .. ..	13 (12)	4.57 (4.55)	11.35 (11.90)	6.78 (7.35)
5.00 to 5.99 .. ..	10 (9)	5.44 (5.48)	10.80 (11.55)	5.36 (6.07)
6.00 to 6.99 .. ..	2	6.15	12.78	6.63

The total mean improvement is 6.35 grammes.

The figures in brackets are calculated after omitting 4 'unsatisfactory' cases.

The first impression given by the above figures is that the degree of improvement is fairly constant, and that the final level of hæmoglobin reached is dependent on the initial level.

As this is an important point we decided to calculate from the individual readings the coefficient of correlation between the initial hæmoglobin and the degree of improvement, and between the initial hæmoglobin and the final hæmoglobin level. We, however, considered that the cases in the unsatisfactory group (Kharikatea—outdoor) should also be excluded and the coefficients of correlation ( $r$ ) have been calculated in the remaining 33 cases; they are, respectively,  $r = -0.301$  and  $r' = +0.460$ ; that is, there is a negative correlation between the initial level of hæmoglobin and the improvement during treatment, and there is a positive correlation between the initial and final hæmoglobin levels, the latter correlation being the higher. This means that there is a greater tendency for the hæmoglobin to increase by a fixed amount, but that there is also some limiting influence which comes into force when the higher levels of hæmoglobin are reached, so that those bloods with a lower initial hæmoglobin level have greater scope for increase.

*The immediate effect of the iron treatment and the maintenance of improvement.*—

Three or four examinations were made during the course of the iron treatment, and in every case a complete examination was made about one month (from 28 to 32 days) from the beginning of the iron course. A final examination, of which we have already analysed and shown the results, was made two or three months later. Between the count at the end of the iron treatment and the final count, the 4 'unsatisfactory' cases being excluded, 20 showed a further increase in hæmoglobin, 9 a decrease, and 6 no change; in 2 cases the coolies were not available when the other final counts were done. The mean of the further increase in these 35 cases was 0.44 g.

In 22 cases an additional count was done one month after the conclusion of the iron treatment; of these 19 showed an increase, 1 no change and 2 a slight decrease. The mean increase in these 22 cases was 0.91 g. At a final

examination in 8 of these cases, one month later, there had been a further increase in 6, in 1 there was no change, and there was a slight fall in the other; in the 8 cases there was a mean increase of 0.72 g. At the final examination in the other 14 of these cases, two months later, only 2 showed a further increase, 2 showed no change, and 10 showed a decrease; the mean difference between the final count and the last count but one in the series was a 0.54 g. decrease. Thus, in this last group of cases, the hæmoglobin appeared to rise for a month to two months after the conclusion of treatment and then it started to fall again.

*The red-cell count.*—According to their final red-cell count the cases can be grouped as follows:—

TABLE XII.

Red-cell count in millions per c.mm.	Males.	Females.	TOTALS.
1.50 to 1.99 ..	1*	1*	2
2.00 to 2.49 ..	..	1*	1
3.00 to 3.49 ..	1*	1	2
3.50 to 3.99 ..	5	3	8
4.00 to 4.49 ..	2	1	3
4.50 to 4.99 ..	1	5	6
5.00 to 5.49 ..	3	6	9
5.50 to 5.99 ..	4	5	9
6.00 to 6.49 ..	1	..	1

\* These are the 4 cases classed as 'unsatisfactory'.

If the 4 'unsatisfactory' cases be excluded, the mean of the whole series is 4.93 millions, of the males 4.97 millions and of the females 4.89 millions.

*The mean corpuscular values.*—The mean corpuscular values at the end of treatment are shown in the table below:—

TABLE XIII.

	Males.	Females.	TOTALS.
Mean corpuscular hæmoglobin (MCH) in $\gamma\gamma$ ..	23.00	23.20	23.11
Mean corpuscular volume (MCV) in cubic $\mu$ ..	74.72	74.24	74.45
Mean corpuscular hæmoglobin concentration (MCHC) per cent.	31.78	31.85	31.82

These figures indicate a decided movement in all the values towards the normal figure. The sex difference seems to have disappeared entirely. Only in the MCHC do they, however, approximate to the normal. A distribution curve of the MCHC values shows a marked concentration round the 31—33 neighbourhood with only isolated incidences below this figure; of the 4 cases below 26 per cent 3 are amongst the 4 'unsatisfactory' cases referred to above and in the fourth there was possibly an error in technique, as in two previous post-treatment examinations the MCHC had been 32 and 29, respectively.

There is thus a deficiency of a little less than 20 per cent in both the hæmoglobin content and the size of the cells, but the saturation of the cells has returned to a figure well within the normal range.

*Reticulocytes.*—As the gardens were some distance from the laboratory it was not possible to do daily counts, so that in the majority of cases the peak of the reticulocyte rise will have been missed. The examinations were done in different cases from the eighth to the eleventh day, but the majority were done on the ninth or tenth day of iron treatment.

The findings can be tabulated as follows :—

TABLE XIV.

*Showing distribution of cases according to their maximum reticulocyte response.*

Maximum reticulocyte response.	Males.	Females.	TOTALS.	Mean initial hæmoglobin level of group to one place of decimals, in grammes.
2 per cent but less than 3 per cent ..	..	1	1	5.5
3 " " " " " 4 " " "	1	..	1	6.2
4 " " " " " 5 " " "	2	3	5	5.3
5 " " " " " 6 " " "	2	2	4	4.0
6 " " " " " 7 " " "	2	2	4	3.8
7 " " " " " 8 " " "	4	1	5	3.8
8 " " " " " 9 " " "	..	4	4	3.8
9 " " " " " 10 " " "	1	..	1	2.4
10 " " " " " 11 " " "	1	1	2	4.0
11 " " " " " 12 " " "	1	1	2	3.9
12 " " " " " 13 " " "	1	1	2	3.9
13 " " " " " 14 " " "	..	2	2	4.0
14 " " " " " 15 " " "	..	1	1	3.4
16 " " " " " 17 " " "	..	2	2	3.2
22 " " " " " 23 " " "	..	..	1	4.7

The 4 'unsatisfactory' cases have been excluded from the table; none of them showed a response higher than 6 per cent.

The reticulocyte response was lower than one would have expected, but this must be due mainly to the fact that in most cases the peak was missed. There is little indication of a relationship between the reticulocyte rise and the initial hæmoglobin level, though the 7 cases showing the least response were certainly amongst the cases with the highest initial hæmoglobin. (Further reference will be made to the reticulocyte responses on the different gardens.)

In 26 cases the reticulocytes fell to below 1 per cent at the final count. This is lower than the average count for the 'normal' coolie of this population. The remaining 15 include the 4 'unsatisfactory' cases. In 3 cases only was the final count about 2.6 per cent and 2 of these were 'unsatisfactory' cases.

The mean initial reticulocyte count in the whole series was about 4 per cent with a maximum of about 10 per cent. This degree of reticulocytosis, whilst being a little higher than that usually associated with hookworm infection, is not of the degree that one gets in hæmolytic anæmias and can probably be explained on the grounds of the stimulation of anoxæmia. The fact that the count in the vast majority fell below the level usually found in the 'normal' coolie population suggests that the hæmopoietic system in these cured anæmics was in a more balanced state of equilibrium than it is in the so-called 'normal' coolie.

*Van den Bergh reaction*—The results after treatment are shown in Table VI. The difference between the percentage of positive findings before treatment is just on the significance level, and the latter are not significantly different from the positive results in 'normal' coolies, shown in Table I.

*The effects of anthelmintic treatment.*

The treatment given was that which was usually employed on the garden in question. The drugs used were oil of chenopodium and tetrachlorethylene; from one to three treatments with one of these drugs or with a combination of the two were given. The efficacy of the anthelmintic treatment given is not a point we propose to discuss here.

The load of infection after treatment is shown in the table below:—

TABLE XV.

<div> <div>Group before treatment</div> <div>Number in group</div> <div>Group into which they fall after treatment</div> </div>			(a)	(b)	(c)	(d)	Grouping after treatment.
			21	10	6	4	
(a)							
10,000 +	..	..	8	1	..	1	10
(b)							
5,000 +	..	..	1	..	1	1	3
(c)							
2,000 +	..	..	4	4	2	1	11
(d)							
200 +	..	..	8	3	1	1	13
(e)							
200 -	..	..	..	2	1	..	3
Not done	..	..	..	..	1	..	1

*Hookworm infection and progress under treatment.*

In column 5 of Table IX the relationship of the initial hookworm egg-count to the progress under treatment is shown.

The mean of the hæmoglobin improvement in the series in which there were more than 10,000 eggs per gramme of stool was 5.74 g. and in that in which there was less, 7.10 g.; the standard errors of the means in the two series are respectively  $\pm 0.6585$  and  $\pm 0.3528$  and of the difference  $\pm 0.7470$ . The difference, 1.36 g., is thus just short of the significance level.

But, if we take an arbitrary division at 4 g. improvement, it will be seen that every patient who failed to improve by 4 g. had an initial degree of hookworm infection represented by at least 10,000 eggs per g. The difference in the percentage of cases that show improvement above this figure (4 g.) in the two groups, above and below 10,000 eggs, is definitely significant.

In considering the egg-counts after treatment, in order to include a slightly larger number of cases we have taken 5,000 eggs as the point of division. The distribution of these cases is shown in column 6 of Table IX.

The mean of the hæmoglobin improvement in the heavily infected group is 5.50 g. and in the rest 6.79 g.; this difference is again not quite significant. It will be seen, however, that 5 out of 6 cases that showed an improvement of less than 4 grammes had 5,000 or more (10,000 in four cases) eggs per gramme of stool at the end of the period of observation. Again, the difference in the percentage of those that showed more than 4 g. of improvement in the two post-treatment hookworm infection groups is definitely significant.

Thus, there is some evidence that the initial load of hookworms has some influence on the progress of the patient under iron treatment, though many of the patients with a heavy load progressed very favourably. There is also evidence that getting rid of the infection has some effect on the progress; although in 6 cases in which a heavy infection persisted the increase was more than 7 g. (51 per cent), of the cases in which there was little progress nearly all were cases in which the infection had persisted.

*Other possible factors in prognosis.*

The mean improvement in 11 cases that showed achlorhydria or hypochlorhydria was 6.91 g.; this figure is slightly higher than the mean of the whole series.

In the 15 cases in which the van den Bergh indirect test was positive before treatment, the mean improvement was 7.43 g. and the mean in 26 negative cases was 5.73; in 7 cases in which the van den Bergh was positive at the end of treatment the mean improvement was 6.18 g.

In the 13 cases with an enlarged spleen the mean improvement was 5.64 g.

Of these the only significant departure from the mean of the whole series is in the case of the pre-treatment van den Bergh-positive cases.

## PROCEDURE ON DIFFERENT GARDENS.

On each of the five different gardens on which we undertook the treatment of these cases, we adopted different procedures. Our original intention was to have six groups, each consisting of 8 cases, but we had to modify our plans on account of shortage of suitable cases. In three groups we had only 6 cases.

The experiment was commenced with 42 cases, but, as already noted, 1 coolie absconded after the first examination and her data have not been included in any of the tables or other calculations; 2 others were not available for the final examination, but the rest were kept under observation for the whole period of 3 or 4 months.

*Excluded cases.*—As we were making a comparison of the different procedures that we adopted and as the numbers in each group were small, we felt that we ought to exclude certain cases in which the response to treatment was influenced by exceptional circumstances. The reasons for the exclusion in each of the 4 cases are given below:—

- (a) Male, aged 24, Nagadholie garden. He was addicted to alcohol and did not give up his habits during treatment; he had achlorhydria. His progress under treatment was not entirely unsatisfactory, as he showed a slight reticulocyte response (7.9 per cent) and his hæmoglobin improved by 4.00 g. in the first month of iron treatment. He was a normocytic case but hypochromic and obviously a case of iron-deficiency anæmia.
- (b) Female, aged 35, Kharikatea garden, hospital case. She was a microcytic-hypochromic case. She showed no reticulocyte response and improved only by about a gramme during treatment, but by the final examination her hæmoglobin was back to its original figure. She had a very heavy hookworm infection; anthelmintic treatment produced no improvement in this. This was obviously not an uncomplicated iron-deficiency case.
- (c) Male, aged 40, Kharikatea garden, out-patient. He was an alcoholic and a very troublesome coolie. His habits could not be controlled as he was an out-patient. There seems some doubt if he retained the iron tablets. He showed no reticulocyte response and his total improvement was just over a gramme.
- (d) Female, aged 30, Kharikatea garden, out-patient. A microcytic-hypochromic case, she responded to iron treatment at first, showing an increase of 3.64 g. at the end of a month, but without reticulocyte response. Within a week the hæmoglobin fell by nearly a gramme and after another 2½ months it had fallen to 0.9 g. below its original level. She had a very heavy hookworm infection that was unaffected by treatment. She was undoubtedly a case of iron-deficiency anæmia, but there must have been some other factor causing the rapid recurrence. It is doubtful if the hookworm infection alone could account for this.

*Nagadholie (Chart 11).*

There were 6 females and 1 male in this series, 1 male having been excluded. They were all hospital patients. The mean hæmoglobin of the group was 4.4 g.

*Dietary treatment.*—The patients were kept on a special diet consisting of milk, eggs, chicken or mutton, sugar, and oranges daily, with fish occasionally, in addition to their ordinary basic diet of rice, dāl, vegetables, and mustard oil (*see Appendix*). The total caloric value of this diet was about 3,500. Of these about four hundred were from protein (half of animal origin), eight hundred from fat (one-third of animal origin), and the rest from carbohydrate. Cod-liver oil and malt was given, a teaspoonful a day during part of the time.



# NAGADHOLIE.

Per cent reticulocytes - - -  
 Per cent Hb. (Helge) ———  
 Millions R.B.C. ———

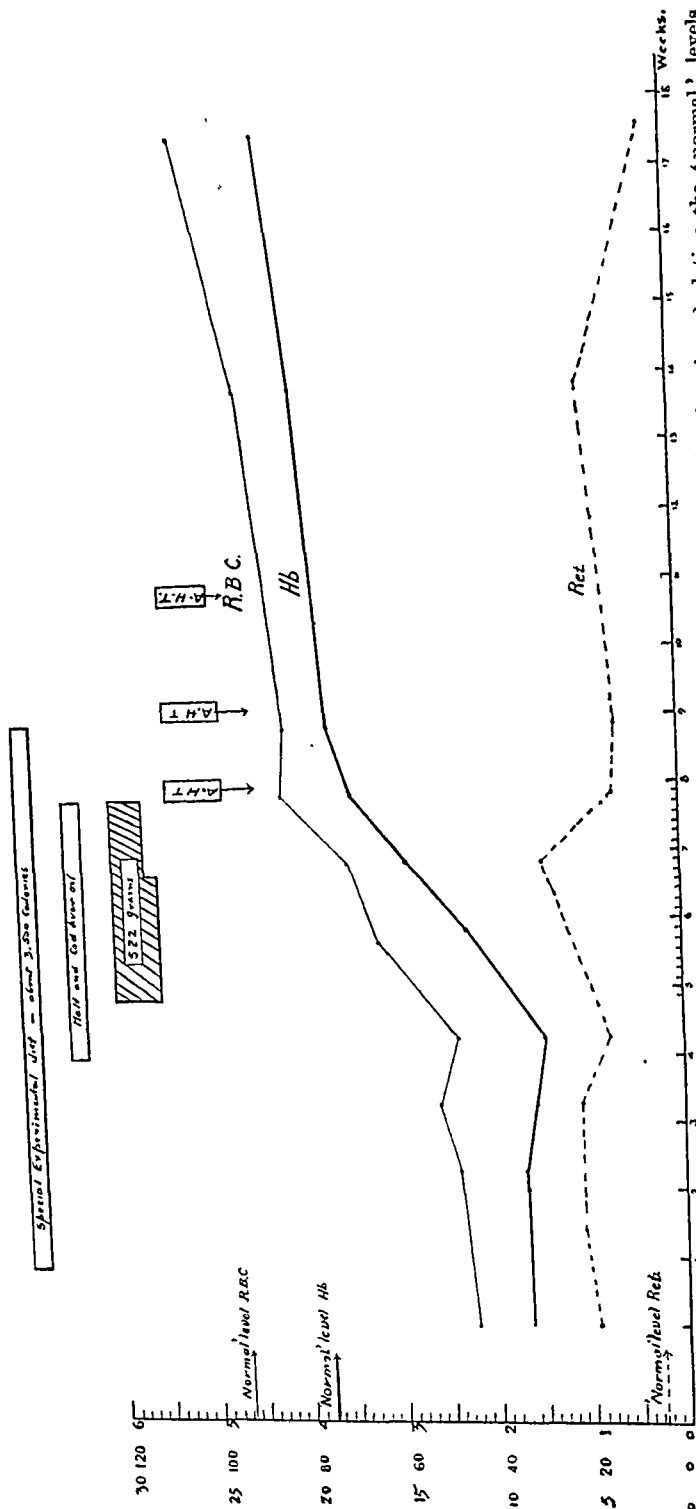


CHART 11. Showing composite curves based on the findings in 7 cases, 1 male and 6 females; in calculating the 'normal' levels in this and the subsequent figures the sex composition of the group has been taken into account.

For the first 28 days this dietary was given; cod-liver oil was added, during the last week, but no iron was given; during this period three blood counts (subsequent to the initial count) were done in each case.

*Response.*—No improvement occurred in the blood picture in any case and the mean hæmoglobin had fallen from 4.4 g. to 4.0 g. at the end of the month. The weight remained constant or there was a slight decrease; however the general condition of the patients was much better and some had lost their puffiness.

*Iron treatment.*—The diet was continued for another month and two teaspoonfuls of cod-liver oil were given twice daily; in addition 27 grains of ferrous sulphate was given daily for the first fortnight and 18 grains daily for about another week. The total amount actually given to each patient was 522 grains.

*Response.*—In every case the response to treatment was immediate and the mean hæmoglobin in the 7 cases was 10.1 g. at the end of 28 days from the commencement of the iron treatment.

*Hookworm treatment.*—The special diet was discontinued and three doses of oil of chenopodium were given at 10-day intervals.

*Progress.*—A count was done after an interval of one month from the last count and the mean hæmoglobin was found to have increased to 10.9 g. At a final count one month later the mean hæmoglobin percentage was 11.7 g. There was a mean increase of four pounds in the weight of the patients during the four months of observation.

### *Murmur* (Chart 12).

In this series there were 3 males and 5 females; they were all treated as hospital patients. The initial mean hæmoglobin level was 3.9 g.

*Diet.*—They were placed on a diet very similar to the one in the Nagadholie group. The caloric value of the diet was also about 3,500, and fat-protein-carbohydrate proportions were the same as in the previous group. Two teaspoonfuls of malt and cod-liver oil were given twice a day. This was given for 38 days, and then the patients were given normal hospital diet.

*Iron.*—From the ninth day of observation a dose of 6 grains of ferrous sulphate was given three times a day for 30 days, a total amount of 540 grains.

*Response.*—In every case the hæmoglobin increase was immediate, but in the majority there was no marked reticulocyte increase on the 17th day (eighth day of iron treatment) when the first post-treatment count was done.

On the tenth day (first day of iron) the hæmoglobin had undergone no appreciable change (the chart shows a slight rise, which is a drawing error). On the thirty-eighth day the mean hæmoglobin had risen to 10.3 g.

*Hookworm treatment.*—On the thirty-ninth and the fifty-third days each patient was given anthelmintic treatment, 1 c.c. of oil of chenopodium and 3 c.c. of tetrachlorethylene.

*Progress.*—On the seventy-fifth day, 66 days after the commencement of the iron treatment, the mean hæmoglobin had risen further to 11.8 g. but at the final count two and a half months later it had fallen to 11.3 g.

CHART 12.

# MURMURIA.

Per cent reticulocytes  
 Per cent Hb. (Hellige)  
 Millions R.B.C.

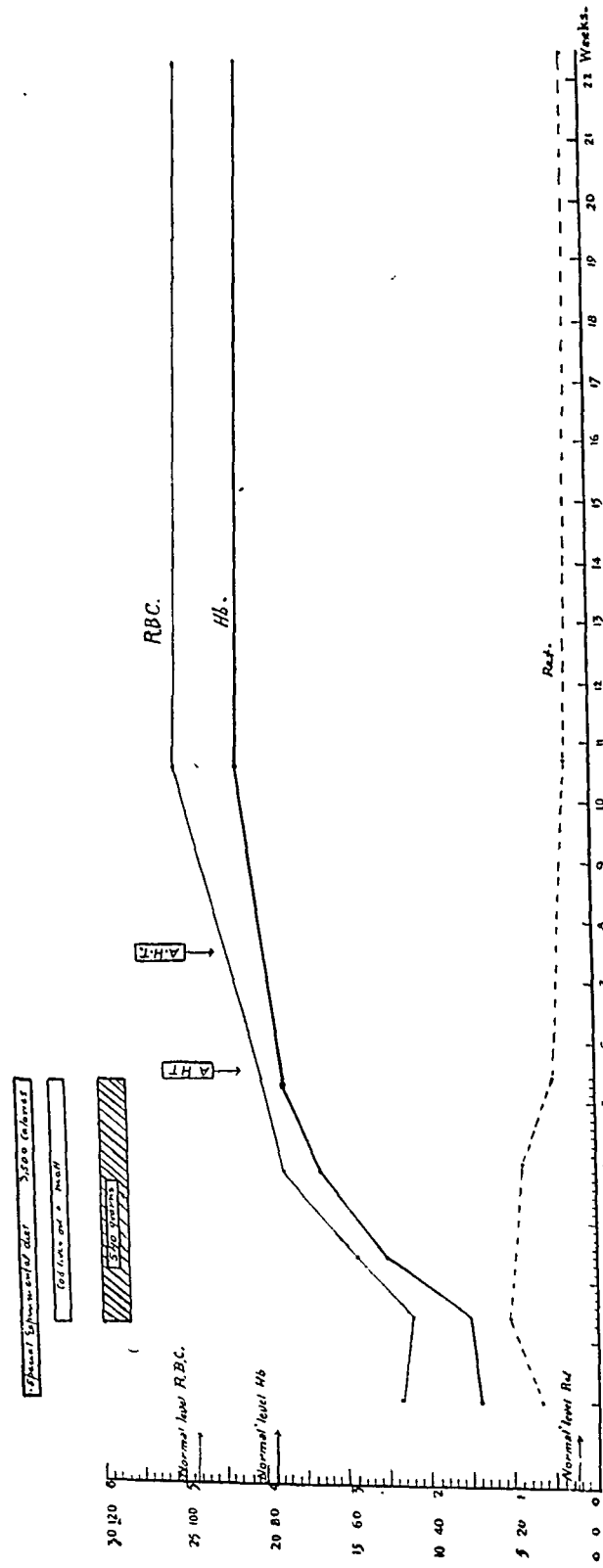


CHART 12. Showing composite curves based on the findings in 8 cases, 3 males and 5 females.

*Weight.*—At the end of the iron treatment 5 had gained in weight and 3 had lost weight: 1 patient lost 7 lb. The mean increase of the group was  $\frac{3}{4}$  lb.

At the end of the period of observation all but 2 had gained in weight and the mean increase of the group was 3·375 lb.

*Sycolla* (Chart 13).

There were 4 females and 2 males in this group; they were all hospital patients. The mean hæmoglobin in the series was 5·1 g.

*Diet.*—No special diet was given to these patients; they all had the usual hospital diet which contains a relatively low proportion of protein and has a caloric value of about 2,200. No cod-liver oil was given.

*Iron.*—They were given 18 grains of ferrous sulphate in three daily doses for 20 days, a total of 360 grains.

*Response.*—In every case there was a marked rise in the hæmoglobin level, but in only 1 case was a reticulocyte rise noted on the eighth day of iron treatment. On the thirtieth day of treatment the mean hæmoglobin in the 6 cases was 11·5 g.

*Hookworm treatment.*—Anthelmintic treatment was given immediately the iron treatment had been stopped. The same dose of oil of chenopodium and tetrachlorethylene was given, but only on one occasion.

*Progress.*—At the end of another month the mean hæmoglobin level had risen to 11·7 g. but two and a half months later it had fallen to 11·0 g.

*Weight.*—Three gained in weight at the end of the first month and 3 remained stationary—mean gain 3·17 lb. At end of observation all but 1 had gained and the mean gain was 7·17 lb.

*Katunibari* (Chart 14).

There were 4 females and 3 males in this group; one patient had absconded after the first examination. They were all hospital patients. The initial mean hæmoglobin level was 4·6 g.

*Diet.*—No special diet was given but the hospital on this garden gives a particularly good diet amounting to 2,500 calories and containing a good proportion of protein. Two teaspoonfuls of malt and cod-liver oil were given twice a day for a month.

*Hookworm treatment.*—In this instance the anthelmintic treatment was given first, one dose of oil of chenopodium and tetrachlorethylene.

*Iron.*—A dose of  $5\frac{1}{2}$  grains of ferrous sulphate was given three times a day for 29 days, a total of  $478\frac{1}{2}$  grains.

*Response.*—Most of the patients showed a slight reticulocyte rise on the tenth day. All showed a marked hæmoglobin increase. On the 29th day the mean hæmoglobin percentage was 11·4 g.

CHART 13.

# SYCOTTA.

Per cent reticulocytes  
 Per cent Hb. (Hellige)  
 Millions R.B.C.

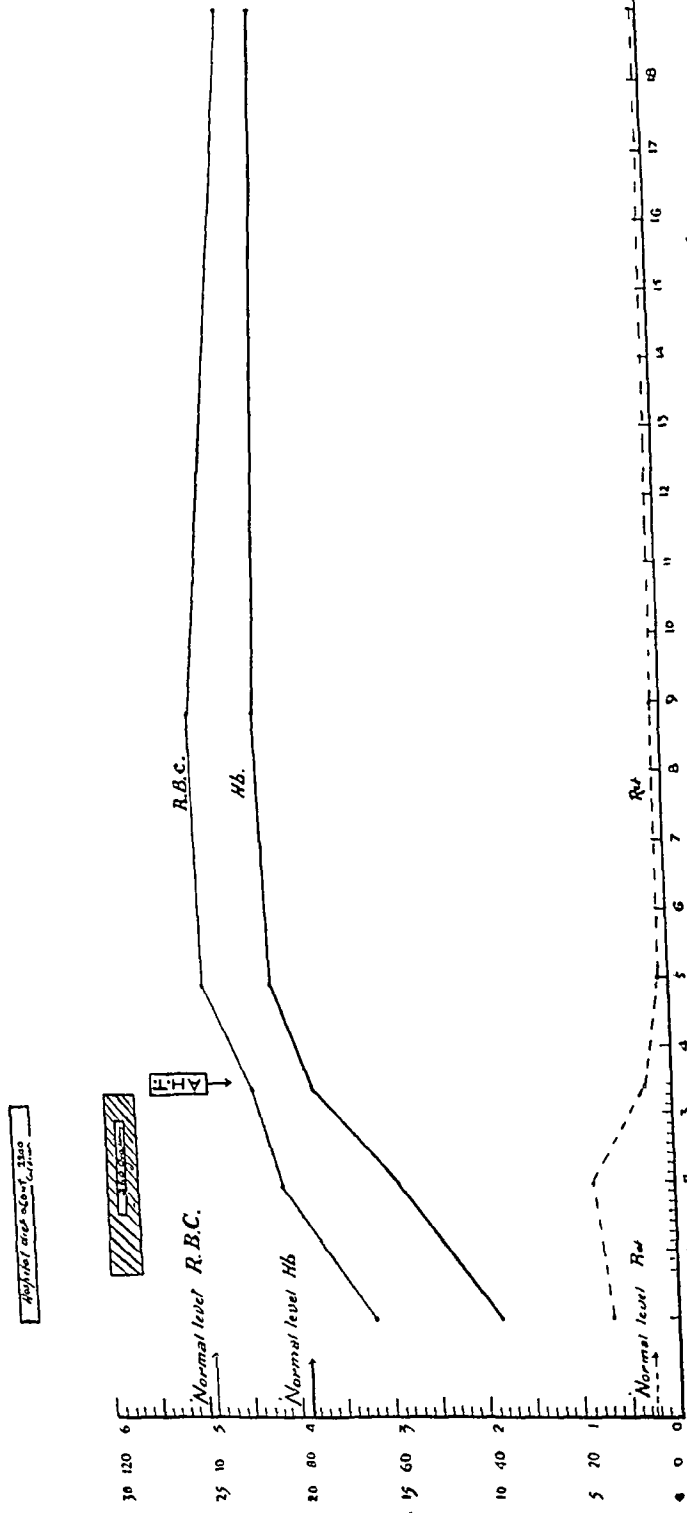
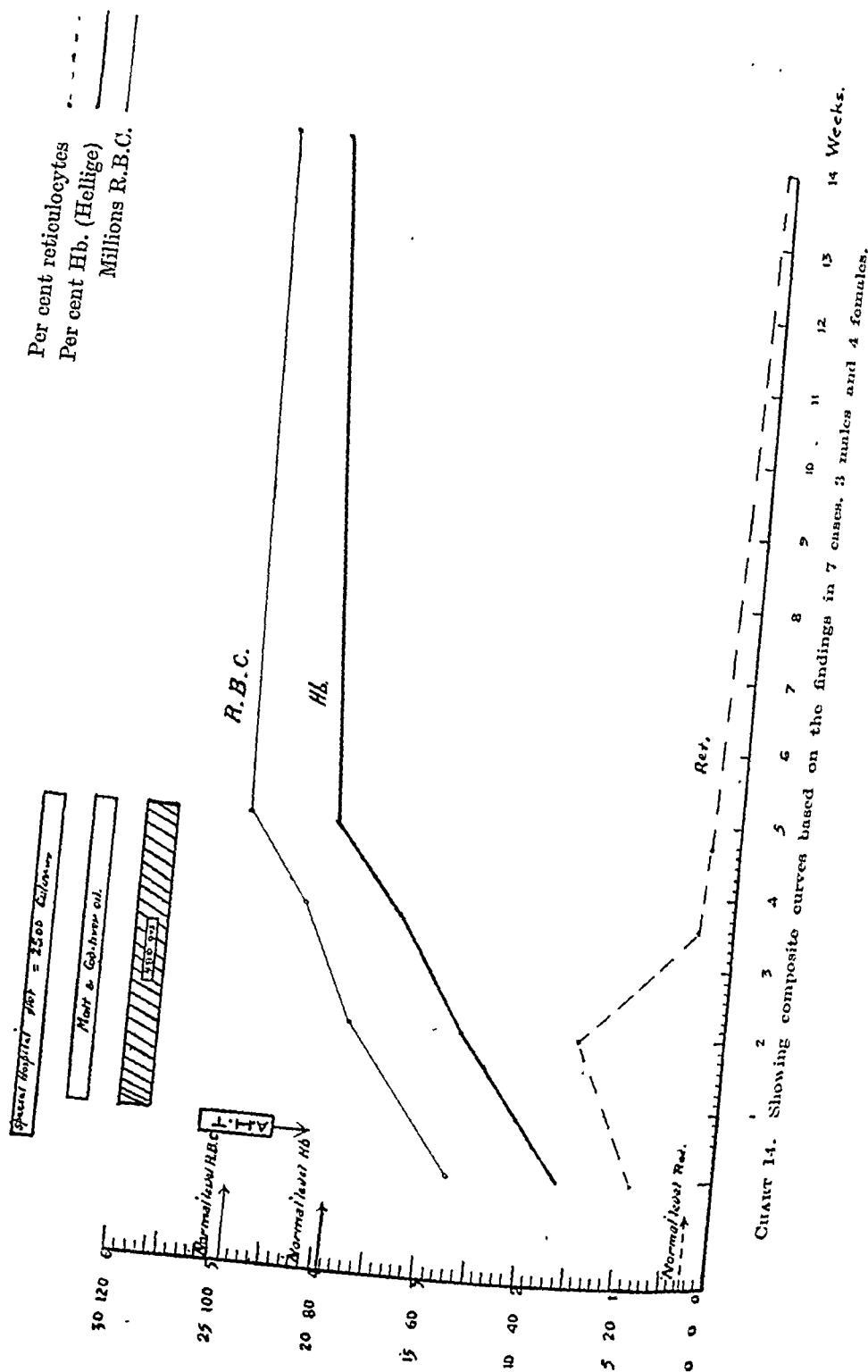


CHART 13. Showing composite curves based on the findings in 6 cases, 2 males and 4 females.

CHART 14.

## KATUNIBARI.



*Progress.*—Sixty-four days later the mean hæmoglobin had risen further to 13.0 g.

*Weight.*—Only 1 patient had gained weight at the end of a month and 4 had lost weight—mean loss 1.83 lb.

Two had gained weight at the end of observation and the mean gain was nearly a pound.

*Kharikateu—Indoor (Chart 15).*

There were 4 males and 1 female in this group. They were all hospital patients. The mean hæmoglobin of the group was 4.2 g. before treatment.

*Diet.*—The patients were on the ordinary hospital diet, of about 2,200 calories. They were given two teaspoonfuls of malt and cod-liver oil daily for three weeks.

*Iron.*—They received a dose of  $5\frac{1}{2}$  grains twice daily for three weeks, amounting to 231 grains.

*Response.*—At the end of 32 days the mean hæmoglobin was 10.2 g. Most of the cases showed a distinct reticulocyte response on the tenth day.

*Hookworm treatment.*—A dose of oil of chenopodium and tetrachlorethylene was given immediately the iron treatment was finished.

*Progress.*—Three months from the conclusion of iron treatment the mean hæmoglobin was 10.0 g.

*Weight.*—Three lost and 2 gained in the first month but the balance was a mean increase of 1.8 lb. One was not weighed again, 1 lost, 1 remained the same and 2 showed a gain at the last examination—mean total gain 4 lb.

*Kharikateu—Outdoor (Chart 16).*

There were 3 males and 1 female in this group. They were treated as dispensary cases and continued to work; they received one teaspoonful of malt and cod-liver oil daily but had no food from the hospital.

The initial mean hæmoglobin level was 4.0 g.

*Iron.*—They were given a single dose of 11 grains of ferrous sulphate before going to work daily for 25 days, a total of 275 grains.

*Response.*—A reticulocyte response was found in every case on the tenth day. The mean hæmoglobin rose to 9.1 g. on the thirty-second day.

*Hookworm treatment.*—A single treatment with oil of chenopodium and tetrachlorethylene was given before the iron treatment was commenced.

*Progress.*—About three months after the conclusion of the iron treatment the mean hæmoglobin level had fallen to 7.7 g.

*Weight.*—Two had lost and 2 had gained, both at the end of a month and at the final weighing. The mean weights remained unchanged.

# KHARIKATEA—Indoor.

Per cent reticulocytes  
Per cent Hb. (Hellige)  
Millions R.B.C.

1200 Calories

Normal level R.B.C.

Normal level Hb.

A.H.T.

30

25

20

15

10

5

0

0

0

0

0

0

0

Normal level R.

Ret.

R.B.C.

Hb.

CHART 15. Showing composite curves based on the findings in 5 cases, 4 males and 1 female.

Weeks.



CHART 16.

# KHARIKATEA—Outdoor.

Per cent reticulocytes      - - - - -  
 Per cent Hb. (Hellige)      ————  
 Millions R.B.C.      ————

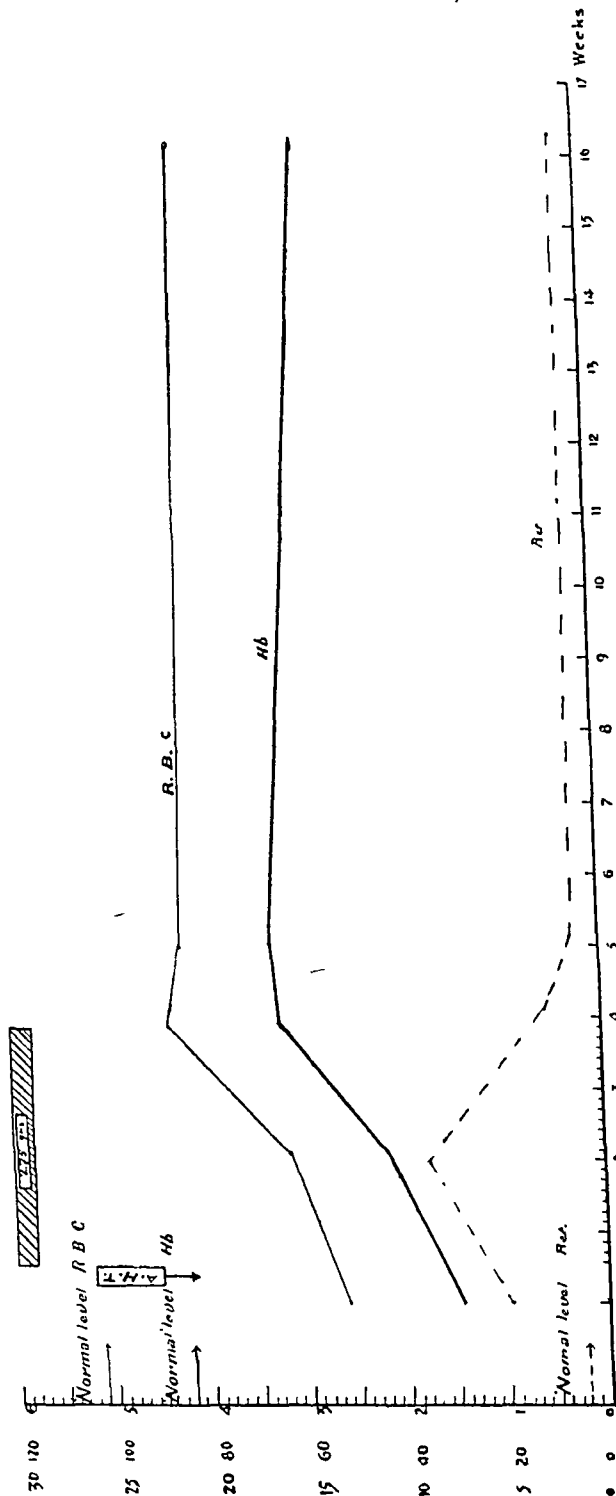


CHART 16. Showing composite curves based on the findings in 4 cases, 3 males and 1 female.

TABLE XVI.

*Summarizing results of treatment on different gardens.*

Group.	Cases.	Diet in calories.	Iron in grains.	ANTHELMINTIC TREATMENT.		MEAN HÆMOGLOBIN LEVEL AND TOTAL INCREASE BEFORE, AND AFTER DIFFERENT PERIODS FROM COMMENCEMENT OF IRON TREATMENT.			
				Before or after iron.	Number of final egg-counts above 5,000.	Before iron.	A month.	About two months.	About three months.
Nagadholie ..	7	Good, 3,500	522	After	1	4.0	$\frac{10.1}{6.1}$	$\frac{10.9}{6.9}$	$\frac{11.7}{7.7}$
Murmuria ..	8	Good, 3,500	540	After	5	3.9	$\frac{10.3}{6.4}$	$\frac{11.8}{7.9}$	$\frac{11.3}{7.4}$
Sycotta ..	6	Ordinary, 2,200	360	After	0	5.1	$\frac{11.5}{6.4}$	$\frac{11.7}{6.6}$	$\frac{11.0}{5.9}$
Katunibari ..	6	Good, 2,500	478½	Before	1	4.6	$\frac{11.4}{6.8}$	..	$\frac{13.0}{8.4}$
Kharikaten—indoor	5	Ordinary, 2,200	231	After	1	4.2	$\frac{10.2}{6.0}$	..	$\frac{10.0}{5.8}$
Kharikaten—outdoor	4	Poor <2,000	275	Before	3	4.0	$\frac{9.1}{5.1}$	..	$\frac{7.7}{3.7}$

*Iron administration in 'normal' coolies.*

In order to see if we could raise the level of the hæmoglobin of 'normal' coolies to that of the normal city-dwelling Indians, we gave in a series of coolies, 4 males and 2 females, a daily dose of 11 grains ferrous sulphate for a period of three weeks—total 231 grains.

From the initial mean level of 11.2 g. the hæmoglobin rose to 12.2 g. at the end of 10 days and then receded slightly and was eventually 12.0 g. at the end of two and a half months.

CHART 17.

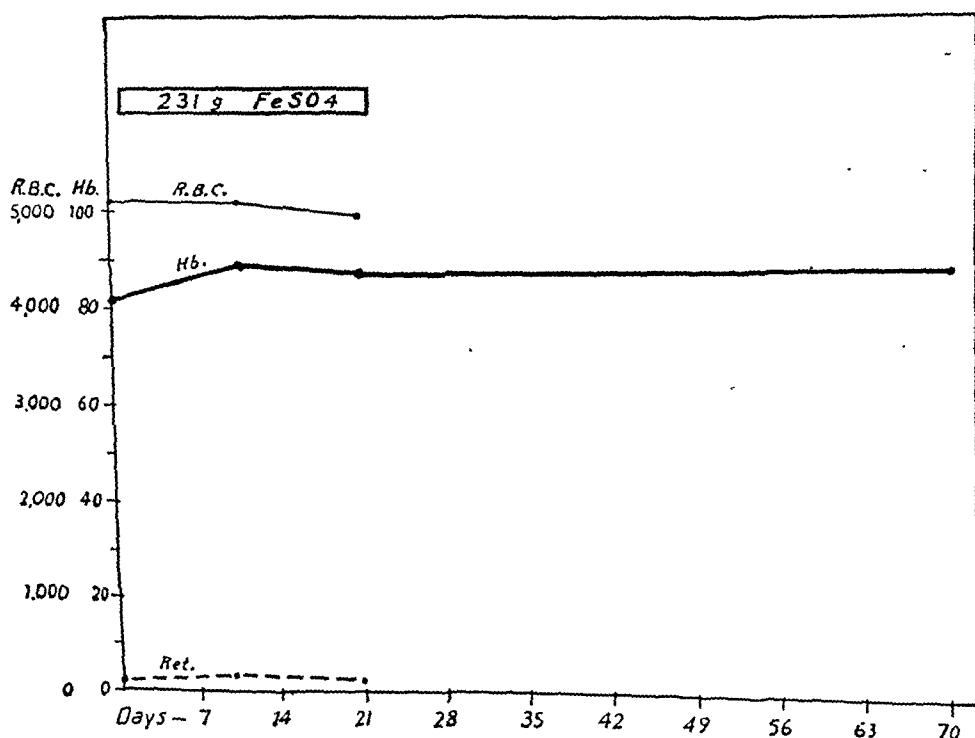


CHART 17. Showing the effect of iron administration to 6 apparently healthy coolies, 4 males and 2 females.

This suggests that there was some slight degree of iron deficiency which was remedied, but that again some limiting factor came into operation before the true normal level was reached.

# APPENDIX.

## PROGRAMME FOR NAGADHOLIE COOLIES DRAWN UP WITH THE AID OF DR. G. MACDONALD.

Substance.	Weight in chittaks.	GRAMMES OF				Carbohydrates.
		PROTEIN.		FAT.		
		Animal.	Vegetable.	Animal.	Vegetable.	
Eggs, two ..	1.42	9.5	..	8.5	..	1.2
*Milk ..	8.0	15.2	..	16.0	..	21.6
†Chicken, half ..	1.5	21.0	..	3	..	..
Rice ..	10	..	38.5	..	3.4	454.0
Dal ..	1	..	11.4	..	0.22	36.2
Potato ..	1	..	1.0	..	..	10.7
Mustard oil ..	1	..	..	..	56.7	..
Onions ..	1	..	0.74	..	0.06	6.3
Cabbage ..	2.7	..	2.0	..	0.2	13.2
TOTALS ..	..	45.7	53.6	27.5	60.6	543.2
GRAND TOTALS ..	..	99.36		88.1		543.2
Calories ..		407		820		2.225

Add fruit—oranges, bananas, or papaya daily.

A large cabbage can be divided amongst six coolies.

\* Milk to be half fresh and half tinned, if the fresh supply is limited.

† The calculation is based on one chicken weighing one pound.

In practice fresh milk was mostly supplied and as mutton was available and cheap, it was frequently given in place of chicken.

## DISCUSSION.

*The nature of the anæmia.*—In our attempt to divide the first series into two separate groups on the hæmatological data we were not supported by statistical evidence, and neither of the only 2 definitely hyperchromic cases showed a high colour index at subsequent examinations. In the second series all but 2 cases were definitely hypochromic, and under treatment these 2 cases came into line with the rest; 32 cases were definitely microcytic. Seven fell within the normal range, and 1 could be classed as macrocytic on the first examination, but of these 8 cases only 1 did not come into line with the rest under treatment.

The patients in both series came from the same population, and, although there were a few cases in the first series with a higher colour index than any in the second series, it seems legitimate to assume that cases in the first series were, as a whole, of the same type as those in the second series about which we have more complete data, especially regarding the response to treatment.

We may, therefore, conclude that with very few possible exceptions the anæmia was of the microcytic-hypochromic type.

In no instance was the anæmia of the pernicious type.

The response to treatment with large doses of iron in 39 out of 41 cases in the second series makes it evident that this microcytic-hypochromic anæmia is an iron-deficiency anæmia.

*The cause of this iron deficiency. Actual deficiency in the diet.*—It is hard to make any estimate of the iron content of the coolies' diet. It is possibly poor in iron but there is strong evidence to suggest that it is richer in iron than that of many non-anæmic vegetarian communities, as the absence of milk and the low fat consumption are the two most striking features in the dietary. The water in the district contains a large quantity of iron, but the iron is in the ferric state.

*Failure of absorption.*—The fractional gastric analyses in 87 cases showed the presence of free hydrochloric acid in 80 cases without the administration of histamine. The incidence of achlorhydria is thus lower than in the normal population and the possibility that the iron deficiency is due to failure of absorption on this account is ruled out.

*Hookworm infection.*—A hookworm infection was demonstrated in nearly every case; in half the cases the hookworm infection was a heavy one. The type of hypochromic microcytic iron-deficiency anæmia from which these patients were suffering is, broadly speaking, the anæmia that is usually associated with hookworm infection.

An examination of random samples of stools taken from the normal coolie population shows almost a hundred per cent infection rate, but only about 14 per cent with a heavy infection (10,000 eggs or more per gramme).

On the other hand, we were unable to show any association between the degree of hookworm infection, as demonstrated by the egg-counts, and the degree of the anæmia.

The actual presence of a heavy infection does not prevent the hæmoglobin from recovering to a point above the normal level of the coolie population, nor

does the persistence of a heavy hookworm infection appear to affect the degree of improvement in individual cases. But in the garden in which the treatment was least effective (Murmuria) the hæmoglobin level tended to fall during the last two months of observation, whereas in another garden (Nagadholie), in which the iron and dietary treatment had been almost the same but the hookworm treatment had been effective, the hæmoglobin continued to rise.

We can summarize our observations by saying that hookworm infection is almost certainly the main factor in the production of anæmia but that, as a normal hæmoglobin level is not incompatible with a heavy hookworm infection, there is probably some other factor that determines why anæmia occurs in some cases and not in others.

*Associated factors in the production of anæmia. Malaria.*—The spleen index on the gardens from which the coolies came varies from 28 to 39 per cent. The spleen was palpably enlarged in about one-third of the cases, but there is no correlation between the splenic enlargement and the degree of anæmia.

In more than a third of the cases there is hyper-bilirubinæmia; this is not a usual finding in simple hookworm anæmia. It suggests, either (a) that there is some liver dysfunction, of which there is no other evidence, (b) that there is excessive hæmolysis, or (c) that the products of normal hæmolysis are insufficiently re-utilized, because of a deficiency in some other substance essential to normal hæmopoiesis.

Excessive hæmolysis suggests concomitant or recent malarial infection, and it does sometimes occur in cases of chronic splenic enlargement; in the first series there was a comparatively high correlation between hyper-bilirubinæmia and splenic enlargement; this correlation was not however 'significant', nor in the second series was there any evidence of such correlation.

Hyper-bilirubinæmia occurs in pernicious anæmia, where it is due to failure of re-utilization of bilirubin, following hæmopoietic dysfunction associated with deficiency of the hæmopoietic principle, but it does not usually occur in iron deficiency; if, therefore, the suggestion (c) above is to be valid there must be some other deficiency. In the second series, the incidence of hyper-bilirubinæmia is considerably reduced following treatment; this fact lends support to the suggestion that it was due to incomplete re-utilization of the products of normal hæmolysis.

The anæmia associated with acute or chronic malaria does not present the picture of the anæmia with which we are dealing; as in malaria there is only intravascular or intracellular destruction of blood, there is not the loss of iron from the body that occurs in hookworm anæmia.

*Diet.*—Though none of the coolies are indigent and all earn sufficient money to provide themselves and their families with an ample diet, there is little doubt that the diets on which they live are ill-balanced and deficient in good protein and in fat; there is little evidence of any vitamin deficiency except possibly of vitamin A.

An experiment was designed to see the effect of diet on the blood picture. Eight patients were kept for a period of 28 days on a well-balanced diet with a caloric value of about 3,500. In no one case did the blood picture improve and the mean hæmoglobin in the series was slightly lower at the end of the period.

These patients were then given large doses of ferrous sulphate for three weeks and there was an immediate response; there was a mean increase of 6 g. in the hæmoglobin of the 8 cases within a period of 28 days.

This experiment shows that dietary re-adjustment by itself will not effect an immediate improvement in the blood picture in cases in which anæmia is established. It does not, however, prove that a deficient diet does not produce a state in which the patient is susceptible to a degree of blood loss that in more favourable conditions would be easily compensated.

We must, however, conclude from the evidence here produced that dietary deficiency is not an important factor.

*The unknown factor limiting the final hæmoglobin level.*—We have shown that, although in nearly all the cases there was a marked response to treatment, there is some limiting factor which prevents improvement beyond a certain level, and that this level is not the level of the better-class, city-dwelling Indian.

Even starting from the higher hæmoglobin level of the 'normal' coolie, improvement beyond a certain point seems to be unattainable.

If we look at the corpuscular values after treatment it will be seen that the MCH and MCV are far below the normal, but that the MCHC approximates very closely to the normal. That is to say, the provision of iron has brought the cell contents up to the usual hæmoglobin concentration, but there is still some factor which interferes with the blood-cell formation.

This deficiency, if it is a deficiency, appears to be common to the whole coolie population as the MCH of the first series of 'normal' coolies (22.2  $\gamma\gamma$ ) was even less than it is in this series.

The main factor in the production of anæmia in hookworm infection is the loss of blood and all its constituents. Iron is obviously a vital constituent and supplying this will bring the blood up to a certain level, but, in the circumstances of the above experiments, not back to normal.

In the anæmia following malarial infection the loss of blood is from the circulation but not from the body. In the process of hæmolysis the iron is saved and stored; we know something of the subsequent course of the blood pigments, but we know little about the fate of the other blood elements, and we do not know whether certain of these, that are not easily synthesized in the body and whose products are not stored, are not broken down during the process of hæmolysis.

Finally, though we have been unable to show that the anæmia can be cured by the provision of a good diet, we cannot be certain that long periods of deficient diet have not reduced the store of some essential factor for normal hæmopoiesis, so that the body is unable to meet the extra demand which is thrown on it by the long-continued blood loss of hookworm infection, and/or by blood destruction in repeated malarial infections.

#### THE RESULTS OF DIFFERENT TREATMENT PROCEDURES.

*The total dose of iron.*—The mean improvement during the first month was fairly constant in the first five groups and bore little relationship to the total iron

dosage, but on the three gardens where the highest dosage was given the improvement was maintained.

The results with the smallest dosage, 11 grains a day for three weeks, were very satisfactory indeed compared with those of treatment by anthelmintic and ordinary iron tonics that was given in the first series.

*The effect of diet.*—The two groups in which a special diet was given were unfortunately those in which the largest dose of iron was also given, but these results were not quite as good as in the Katunibari group where no special diet was given, though the normal hospital diet is a good one. In the Sycotta group, where a very adequate dosage of iron was given and where the effect of hookworm treatment was very satisfactory, there was an early falling off in the hæmoglobin level, and in the Kharikatea-outdoor group, where the diet was unsatisfactory, the results were poor; on the other hand at Katunibari, where the normal diet of the coolies is good, exceptionally rapid progress was made and maintained. The results in these two groups do suggest, but not very forcibly, that diet has some effect on the maintenance of a high hæmoglobin level.

*Hookworm treatment.*—In the first series, where for all practical purposes only hookworm treatment was given, except to those treated as hospital patients, the results were far below the worst results in the second series. It is, therefore, apparent that anthelmintic treatment alone will not effect a cure except possibly after a long interval.

It is quite obvious that almost maximal improvement can be achieved without anthelmintic treatment, and that in the presence of a heavy hookworm load a high hæmoglobin level can be maintained for some months (*vide* Murmura). On the other hand, it is quite certain that in time the hookworm infection will cause a recurrence of the anæmia; for example, in the Kharikatea-outdoor group, where a heavy infection was maintained, there was a marked fall in the hæmoglobin level even during the short period of observation of this experiment.

It seems to be a matter of little importance whether the anthelmintic treatment is given before or after the iron treatment from the point of view of the eventual result, but there are certain advantages in giving the anthelmintic treatment to a healthy rather than to an anæmic individual. In this investigation, some of the best results were achieved by giving the anthelmintic treatment first but so also were the worst results [*vide* Katunibari and Kharikatea—outdoor].

#### SUMMARY.

In 100 cases of anæmia amongst tea-garden coolies there was no case of anæmia of the pernicious type; with very few possible exceptions they were all cases of the microcytic-hypochromic, iron-deficiency type.

The main cause of the anæmia is hookworm infection, but there are almost certainly other associated causal factors; the possibility of these being malaria and dietary deficiency are discussed.

Anthelmintic treatment alone produced little improvement in the anæmia. Dietetic treatment alone also produced no effect on the blood picture.



Large doses of iron (e.g., 18 grains of ferrous sulphate a day for three weeks) caused a rise in the hæmoglobin to the level of the 'normal' coolie population, without any anthelmintic treatment; if effective anthelmintic treatment was then given this level was maintained.

In the iron-treated cases the concentration of the hæmoglobin in the cells reached the normal, but the volume and the hæmoglobin content of the cells still remained about 20 per cent below the normal level, though both values were about equal to those that we have previously shown are the normals for the so-called healthy coolie population.

#### ACKNOWLEDGMENTS.

Our thanks are due to Dr. D. Manson of the Jorehaut Tea Company for placing a laboratory at our disposal, and to both Dr. George Macdonald and Dr. Manson for their active co-operation, without which this work could not possibly have been carried out, and for many very valuable suggestions during the investigations, to the assistant medical officers in charge of the various tea gardens, and to Mr. L. De who rendered valuable material assistance especially in connection with the counting of hookworm ova. Our thanks are also due to the managers of the gardens on which the coolies were employed, particularly Messrs. Syme and Boswell each of whom took an active interest in the investigation on their respective gardens.



## A STUDY OF HUMAN PAROTID SALIVA.

BY

M. A. BASIR, M.B., B.S., PH.D. (Lond.),

AND

T. S. RAMABHADRAN, M.A., L.T.

(*From the Medical College, Vizagapatam.*)

[Received for publication, July 28, 1936.]

IN this paper an attempt has been made to determine quantitatively the relative activity of human parotid saliva with reference to normal mixed saliva.

The parotid saliva for the experiments was from a patient in the wards of the King George Hospital, Vizagapatam, under the care of Captain M. G. Kini. The patient was one Manthi Tata, aged 26 years, Hindu, coolie, married but without children. He had an injury on the right side of the face and neck. Surgical treatment was rendered, but there was a persistent parotid fistula still left. The opening was 2 mm. to 3 mm. in diameter and slightly infected. He had no temperature and was moving about. He was a non-vegetarian, taking ordinary diet, viz., bread, milk, rice, and curry. Whenever he took food, the saliva dribbled from the fistula and it was collected by applying the mouth of a flask over the fistula after rejecting the first flow. About 100 c.c. were collected each time and fresh saliva was used in the experiments.

In this connection we express our grateful thanks to Captain M. G. Kini for kind facilities offered in giving us access to the patient, for the ready and fresh supply of the saliva every day.

### I. PHYSICAL PROPERTIES AND SACCHAROGENIC POWER.

#### (a) *Physical properties.*—

Appearance—slightly opalescent and slimy.

Smell—odourless. Density—1.0064 at 27°C.

Reaction—alkaline—pH 9 to 9.5 using B. D. H. Universal Indicator.



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Reaction—alkaline—pH 9 to 9.5 using B. D. H. Universal Indicator.

(b) *Method*.—The method of estimating salivary activity adopted was that of Sherman and Walker (1921) with slight modifications. The pure saliva was diluted a known number of times with distilled water according to the needs of the experiment (5 times in the case of mixed saliva and 25 times in the case of parotid saliva). The substrate consisted of 100 c.c. of a 1 per cent dispersion of soluble starch, activated with 5 c.c. of 1 molar sodium chloride and 2.5 c.c. of 0.02 molar disodium hydrogen phosphate, and maintained at a constant pH of 6.64 by the addition of 10 c.c. of phosphate buffer, the temperature being maintained at 38°C. to 40°C. One c.c. of the diluted saliva was allowed to act on this for 30 minutes after which its action was arrested by the addition of 50 c.c. of Fehling's solution. The flask was immersed in a boiling water-bath for 15 minutes. The cuprous oxide formed by reduction was estimated volumetrically by the method of Bertrand (1906). The precipitate, after being filtered through special Gooch asbestos washed with hot water, was dissolved in excess of acid ferrie alum and the reduced iron was titrated against standard potassium permanganate.

*Result*.—Saccharogenic power—14.5 g. of cuprous oxide per c.c. of pure undiluted parotid saliva.

(c) The relative activities of different specimens of saliva on different forms of starch were also studied by the same method with the following results:—

*Saccharogenic power in grammes of cuprous oxide per c.c.  
of undiluted sample.*

Sample.	Starch amylum	Starch soluble.
1. Mixed saliva A .	2.325	2.325
2. Mixed saliva B .	1.700	1.730
3. Parotid saliva ..	14.500	14.500

## II. ALLEGED OCCURRENCE OF ACETYLCHOLINE IN HUMAN SALIVA.

Secker (1934) concluded after a series of experiments that the saliva obtained from the sub-maxillary gland of a cat contains a substance resembling very much like acetylcholine in its biological action. Later, Gibbs (1935) repeated Secker's experiments carefully and put forward experimental evidence to show that the substance in the saliva of the cat, which was believed by Secker to resemble acetylcholine, is not acetylcholine itself, but a potent cardiac depressor, the nature of which is still not established.

In the present study with the human parotid saliva, experiments were performed on the frog's heart with a view to determine the nature of the cardiac depressor, if any, alluded to by Gibbs.

### *Experiments and observations.*

A frog's heart was isolated and arranged for perfusion with Ringer's solution, in the usual manner as shown in Fig. 1. Experiments were then performed both with the sample of fresh human parotid saliva and a 1 in 1,000 solution of acetylcholine (acecoline of l'Lematle et G. Boinet, Paris).

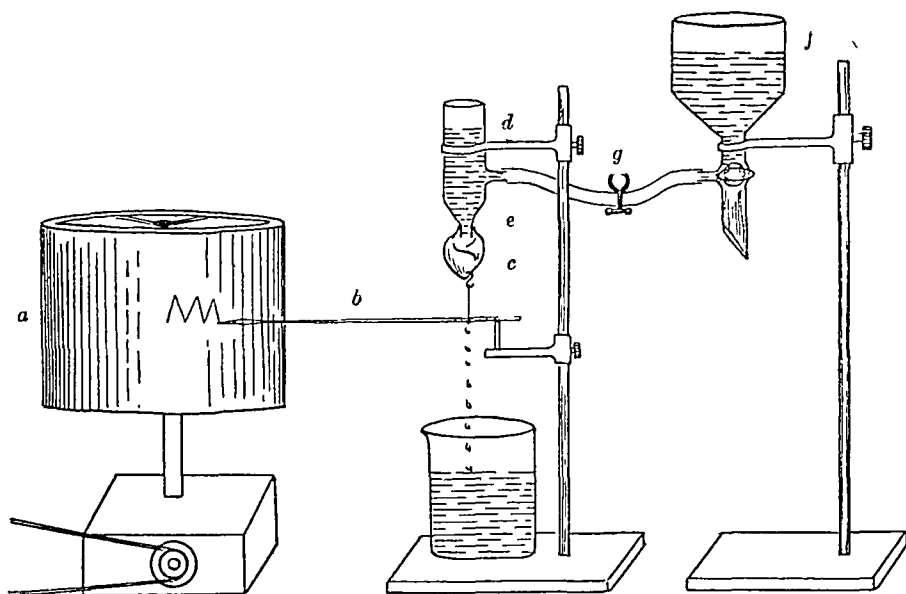


Fig. 1. *Perfusion of isolated frog's heart.*—(a) is the drum on which writes an aluminium lever (b) of Harvard & Co. The lever (b) is attached to an isolated frog's heart (c) by means of a hook. The heart (c) is perfused with frog's Ringer's solution by means of a Syme's perfusion cannula (d) which is tied in the sinus venosus of the heart. The cannula (d) is connected by means of a side tube (e) with a reservoir (f) of the frog's Ringer's solution. The side tube (e) has a pinch-cock (g) to regulate the flow of the Ringer's solution.

#### (1) *Experiments with the saliva.*—

(a) Five drops of fresh human parotid saliva were added to the Ringer's solution directly into the Syme's perfusion cannula (Fig. 1, d). The amplitude of the heart-beat gradually decreased and finally the heart remained in a slightly contracted condition beating somewhat irregularly. After a short interval, the heart began to beat as before as shown in Fig. 2. So it is evident that the human parotid saliva contains a potent cardiac depressor which inhibits the heart in partial systole.

(b) In another similar experiment, the heart was first subjected to the action of atropine (5 drops of 0·5 per cent solution) and then nicotine (5 drops of 1 per

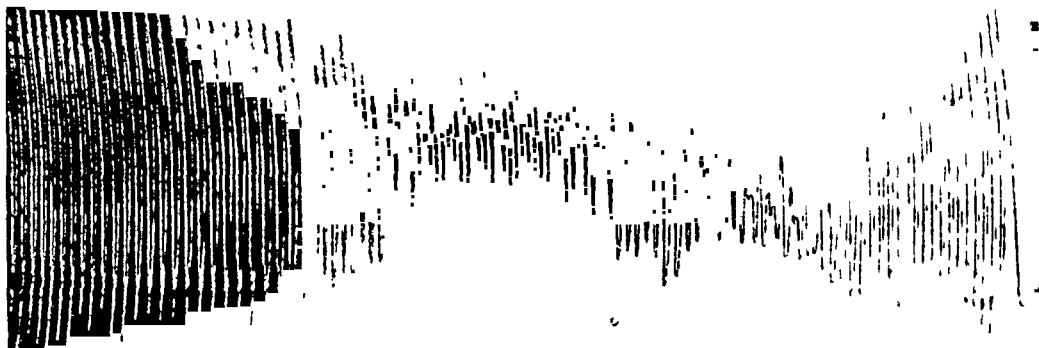


Fig. 2. *Action of human parotid saliva on the frog's heart.*—To be read from right to left. After recording a few normal heart-beats, saliva was added at the mark indicated. The heart was inhibited in partial systole and the beats were small and irregular. Again the heart resumed its normal rhythm.

cent solution) and finally human parotid saliva (5 drops) was added into the Syme's perfusion cannula (Fig. 1, *d*). The results are shown in Fig. 3.



Fig. 3. *Action of the human parotid saliva on the frog's heart, after subjecting the heart first to the action of atropine and nicotine.* To be read from right to left. The heart was inhibited completely in partial systole.

Again the heart was found to be completely inhibited in partial systole. It is at once evident that atropine and nicotine have not modified the action of the cardiac depressor present in the saliva, but on the other hand have accentuated its action.



(2) *Experiments with the solution of acetylcholine (1 in 1,000).—*

(a) To another preparation of frog's heart, as in the above perfusion experiment, 5 drops of acetylcholine (1 in 1,000) were added to the Syme's perfusion cannula (Fig. 1, *d*). The heart was inhibited in diastole as shown in Fig. 4. Then gradually the heart began to beat with the usual rhythm.



Fig. 4. *Action of acetylcholine (1 in 1,000) on the frog's heart.*—To be read from left to right. At Ac, the acetylcholine solution was added. The heart was inhibited in diastole. Gradually the heart regained its usual rhythm.

(b) The same experiment as above was repeated with another frog's heart, but it was subjected at first to the action of atropine (5 drops of 0.5 per cent solution) and then nicotine (5 drops of 1 per cent solution) and then finally acetylcholine (5 drops of 1 in 1,000 solution) was added to the Syme's perfusion cannula (Fig. 1, *d*). As shown in Fig. 5, there was no change in the heart-beat brought about by acetylcholine.

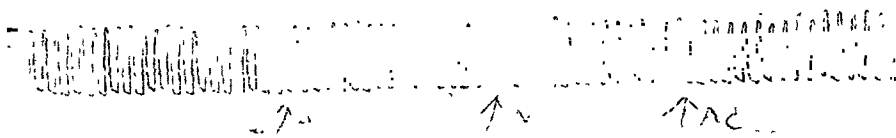


Fig. 5. *Action of acetylcholine (1 in 1,000) on the frog's heart.*—After subjecting the heart at first to the action of atropine and nicotine. To be read from left to right. At A, atropine solution, at N, nicotine solution and at Ac, acetylcholine solution were added in succession. No effect was noticed in the heart-beats.

Evidently then, the preliminary treatment of the heart with atropine and nicotine had annulled the inhibitory action of the acetylcholine. On the other hand, in one of the experiments, there was an actual increase in the rate and amplitude of the heart, showing thereby that the action of acetylcholine can be reversed by such preliminary treatment of the heart with atropine and nicotine.

## DISCUSSION.

The human parotid saliva, therefore, contains a powerful cardiac depressor whose action is not modified by atropine or nicotine (Fig. 3). This depressor

(b) In another similar experiment, the heart was first subjected to the action of atropine (5 drops of 0.5 per cent solution) and then nicotine (5 drops of 1 per

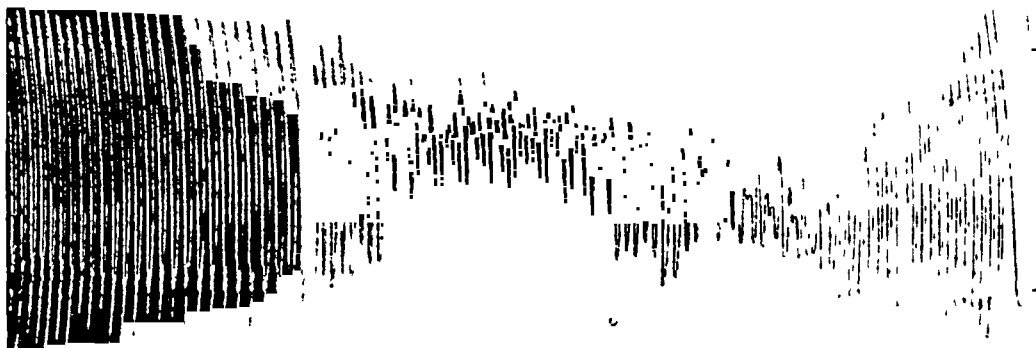


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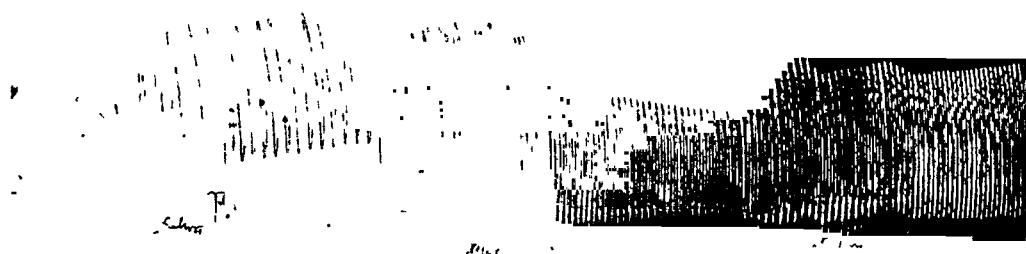


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Again the heart was found to be completely inhibited in partial systole. It is at once evident that atropine and nicotine have not modified the action of the cardiac depressor present in the saliva, but on the other hand have accentuated its action.

## THE POSSIBLE INFLUENCE OF SOLAR RADIATION ON THE PRODUCTION OF CATARACT IN CERTAIN DISTRICTS OF SOUTHERN INDIA: A PRELIMINARY INVESTIGATION.

BY

LIEUT.-COLONEL R. E. WRIGHT, C.I.E., I.M.S.,

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*(Inquiry under the Indian Research Fund Association.)*

[Received for publication, July 20, 1936.]

It has been held for a long time that cataract is much more common in India than in Western countries. The idea that exposure to excessive solar radiation was largely responsible was a natural corollary. Of recent years scientific interest in these views has been stimulated by Vogt's (1931) work on the experimental production of cataract by infra-red (ultra-red) rays. He showed that the near infra-red region (2,400 to 750 millimikra) was very active in this respect, whereas the more distant infra-red and ultra-violet radiations were not damaging to the lens. The well-known cataracts met with in glass-blowers and iron-puddlers are also a product of the absorption of infra-red radiation. These have certain peculiar features some of which are readily recognized. Mulock-Houwer (1934) recorded that lenticular changes which he and Mingelen had observed in Europeans in Java showed a great similarity to glass-blowers' cataract. He found that in the Javanese, cataract of a caloric type is not so common as in Europeans living in Java, and inferred that the lenticular change in the latter is an indication that exposure to great intensities of solar radiation is a definite ætiological factor in persons not protected by heavy iris pigmentation. He suggests that in British India the ultra-red radiation may be more active, and constitutes an important ætiological factor in cataract production even in Indians; especially in areas which, unlike Java, are dry and in which atmospheric moisture does not partially absorb the infra-red radiation reaching the earth's surface.

Long experience of cataractous conditions did not lead me to think that the characteristic features of infra-red absorption were sufficiently common to warrant the view that the part played by thermal energy was important enough in South India to account for an exceptionally high frequency in the occurrence of cataract.

In order to get further information on these matters this investigation here recorded was undertaken.

At the outset one had to assume it probable that solar radiation, (and its infra-red extension with which we are now concerned), reached the earth in greater intensities in hot, dry, cloudless districts, and in lesser intensities in moist and more cloudy areas. This presumption may not be wholly justified since the various atmospheric factors influencing the solar radiation is a problem full of difficulties. It is commonly accepted, however, that aqueous vapour in the atmosphere absorbs a considerable amount of the infra-red radiation and prevents it reaching the earth.

Two districts were chosen, Bellary (Deccan) and Calicut (Malabar), in one of which the outdoor labourer was liable to be exposed to greater intensities of the infra-red solar radiation than in the other. The choice of districts was limited inasmuch as it was necessary to obtain the help of trained observers. In each district some 2,000 outdoor labourers between the ages of 40 and 60 were inspected. They were taken at random and examined for evidences of cataractous change. In the early or incipient cases, posterior sub-capsular change was looked for. The presence of this feature does not furnish certain evidence of a thermal causation, but its absence practically negatives this ætiology.

It was impossible under the conditions of the investigation to have corneal microscope observations made, to determine if the changes in the lens capsule (separation of the zonular lamella), said to be peculiar to caloric cataract, were present; nor yet could it be noted with certainty if the posterior sub-capsular change characteristic of the absorption of infra-red rays was the type actually observed. There are other posterior sub-capsular or saucer-like cataractous changes not due to infra-red absorption with which the former is liable to be confused unless subjected to examination with a corneal microscope. It is possible, however, by ordinary clinical methods to note the presence or absence of posterior sub-capsular changes in the majority of incipient cases and thus determine a group which would include incipient cataracts of infra-red origin. A relative frequency or infrequency of posterior sub-capsular cataract, including all types, might reasonably be held to discriminate for or against the ætiological importance of infra-red radiation.

A statement of the collected figures is given in the Table opposite.

In attempting to draw conclusions from the figures here presented there are a number of points which must be taken into consideration as detracting from their value. Amongst others the following are important:—

- (a) The ages 40 to 60 are presumptive. Few peasants know their age. It may be inferred that the average individual in these groups looked older than his or her actual age. The term senility is not a convenience when considering cataract in India, since it is associated in the minds of many with great age rather than with unknown noxious influences of various sorts in greater or lesser amounts acting over a variable time period. We are apt to speak of premature senility. Such a condition would probably be common amongst the individuals of our groups.

TABLE.  
The occurrence of cataract in outdoor workers between the ages of 40 and 60 in  
South Indian districts.

Place.	Number of persons examined.	IMMATURE OR INCI- PIENT CATARACT.		Mature cataract.	Total cataract.	Incidence of cataract.	Incidence of posterior sub- capsular cataract.	REMARKS.
		Posterior sub-cap- sular.	Other than posterior sub-cap- sular.					
I. DECCAN (Bellary).—								
Average maximum tem- perature, 93.3°F.	2,000	19	276	94	389	1 in 5.1 approx.	1 in 15.5	The figures do not reveal anything likely to be of importance in the rate of occurrence amongst males and females res- pectively in the Deccan group.
Average mean temperature, 82.0°F.								
Relative humidity, 61 per cent.								
Rainfall, 1.6 inches Number of rainy days, 2.6								
II. MALABAR (Calicut).—								
Average maximum tem- perature, 86.7°F.	2,000	5	461	123	589	1 in 3.4	1 in 93.2	In the Malabar group a note of sex was only made in the case of 743. The figures do not suggest any great divergence in the rate of occurrence as between males and females, but the total number of females examined is too small for comparative pur- poses.
Average mean temperature, 80.7°F.								
Relative humidity, 83 per cent.								
Rainfall, 9.75 inches Number of rainy days, 8.6								

The incidence of cataract is very high. It is higher in Malabar where the factor of infra-red radiation is less operative.

The posterior sub-capsular types are more common in the Deccan figures where infra-red radiation may be expected to play a more active rôle.

- (b) In Group II (Malabar) four observers are responsible for the figures, whereas in the other group one observer saw all the cases. Errors of observation are more likely to have been recorded in Group II, but it is improbable that the difference in incidence of posterior sub-capsular cataract can be accounted for in this way to any great extent.
- (c) Of the posterior sub-capsular cataracts in either group, one cannot say with certainty if any are directly due to infra-red radiation.

In spite of various sources of error, the data appear to show :—

1. That the incidence of cataract between the ages stated is unexpectedly high in each group (1 in 5.1 and 1 in 3.4, respectively).
2. It is higher in the area where one might have expected the effects of infra-red radiation to be less active.
3. The occurrence of posterior sub-capsular cataract is very much less common in the area where presumably there is a lesser exposure to that portion of the infra-red radiation known to be absorbed by the lens.
4. Although one cannot conclude that infra-red radiation is a deciding factor in determining the greater frequency of posterior sub-cortical cataract in the area in which it might *prima facie* be expected to be more active, it is significant that this greater frequency appears (1 in 15.5 as compared with 1 in 92.2).
5. The relative infrequency of posterior sub-cortical cataract in the Malabar area appears to show that infra-red radiation is ætiologically unimportant as compared with other causative agents.

This investigation, taken in conjunction with the fact that bio-microscopical observations on posterior sub-capsular cataracts in Madras during the past ten years have revealed few in which characteristic infra-red changes were present, would seem to indicate that in South Indians solar radiation is probably not an important factor in determining the high frequency of cataract which evidently exists.

#### ACKNOWLEDGMENTS.

My acknowledgments are due to the following for collecting the data on which this paper is based :—

Dr. P. Narayana Menon, retired Civil Assistant Surgeon, Mr. D. Narasimha Raju, Sub-Assistant Surgeon, Mr. M. Anandan, Sub-Assistant Surgeon, Mr. P. V. Karunakaran Nair, Honorary Sub-Assistant Surgeon, Dr. P. Balakrishna Menon, and to Lieut.-Colonel Leslie, I.M.S., for his co-operation.

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| VOGT, A. (1931)      | .. | .. | 'Lehrbuch und Atlas Spaltlampen-<br>mikroskopie des lebenden Auges', 2. |

## ON THE MALE TERMINALIA OF *CHRYSOMYIA* *MEGACEPHALA* AND *C. BEZZIANA*.

BY

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(From the Department of Medical Entomology, School of Tropical Medicine,  
Calcutta.)

[Received for publication, July 28, 1936.]

PATTON and CUSHING (1934) have given a comparative description of the terminalia in both the males and females of some of the species of *Chrysomyia*, and these studies have enabled them to compose a reliable practical guide for the classification of the genera. I now wish to review the Oriental *Chrysomyias* in this respect but in this paper observations will be confined to two species *C. bezziana* and *C. megacephala* only, and I intend to state only the salient characters whereby the differentiation between the males of the two species can be effected.

In conducting these observations some difficulty was experienced at first over the features of some flies which resembled *C. megacephala* in all their external characters, except that their colour was a metallic green as against the dull bluish-green of *C. megacephala*, and in which there was also a difference in size and in their natural breeding habits. Earlier too in the investigation it was thought that there was a well-marked difference in their terminalia and it was believed that they represented a new species. But after a large number of these flies had been examined it was found that they were *C. megacephala* and that this species exhibited a great deal of variation in the disposition of bristles on the paramere.

For the preparation of the flies they were treated with caustic potash, cleared in carbolic acid, and then dissected in clove oil. In the descriptions of the different parts Patton's (1932) terminology has been used. All the preparations of the male terminalia have been made in the same position and the drawings executed with the camera lucida (see Plates XXXI and XXXII).

My conclusion is that no appreciable difference can be detected in the character of the 9th coxite and anal cercus or in the shape of the 9th tergo-sternum, and, in the main structural details, the phallosome of *C. bezziana* and of *C. megacephala* resemble each other.

The parameres alone present distinctive characters in the two species. The number of long hairs present on the ventral edge of the anterior paramere varies: in *C. megacephala*, the usual number is 5 but 3, 4, and 6 have been noticed, while in *C. bezziana* the number is constantly 4, so that this feature itself is not invariably diagnostic.

The position of the attachment of the sensory spines on the posterior part of the paramere varies widely in *C. megacephala*, as will be evident from the illustrations. The first spine on the accessory process is usually placed at the junction of the outer third and inner two-thirds, it projects out considerably from the body and is placed on a well-marked pedestal. In *C. bezziana*, on the other hand, it is placed much lower down, at the concavity at the junction of the accessory process and the shaft of the posterior part of the paramere. All the spines are considerably better developed in *C. megacephala* than in *C. bezziana*.

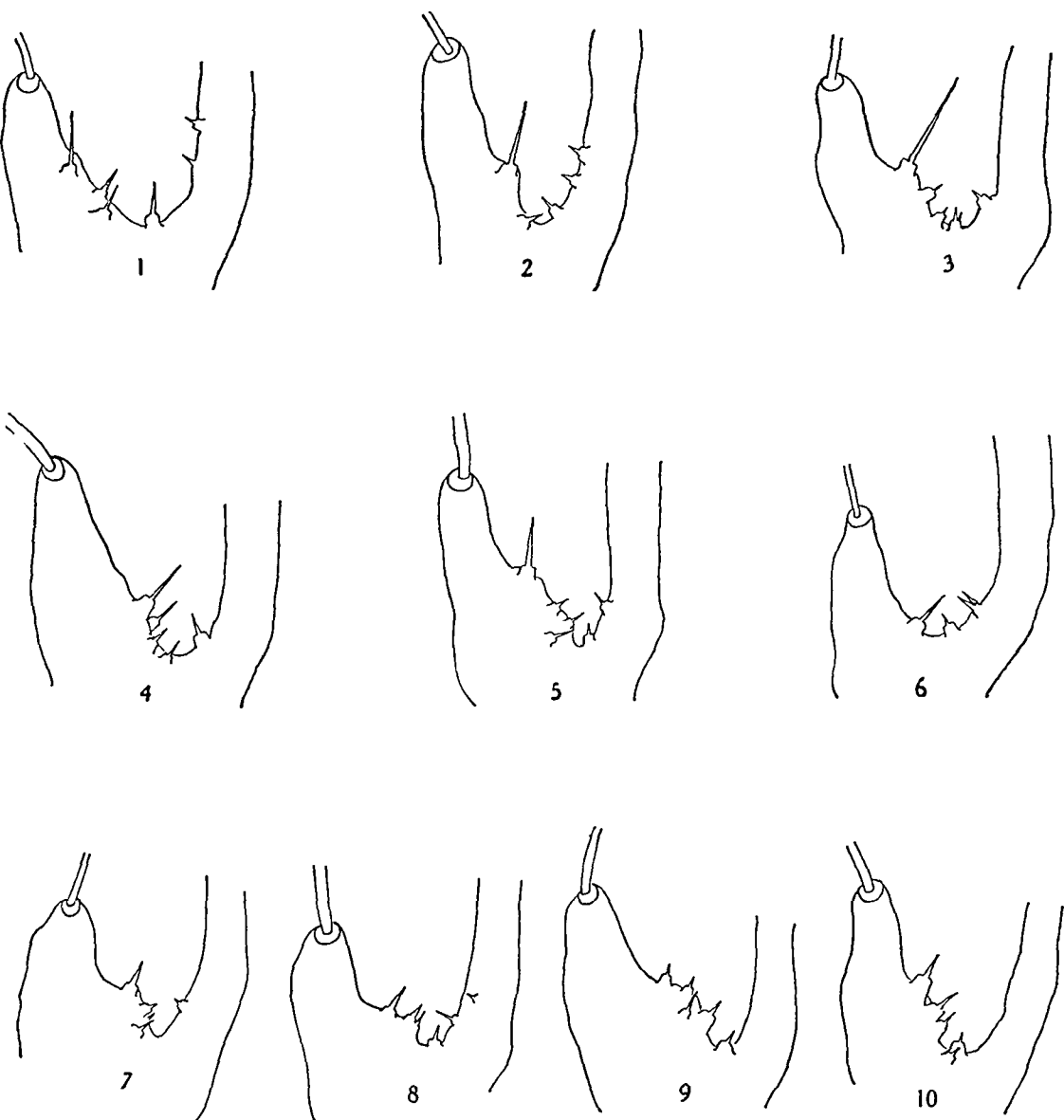
It will thus be seen that an intensive study of the male terminalia of higher diptera, along the same lines as Patton, is likely to reveal characters of 'specific' distinction, a matter which was first pointed out by him.

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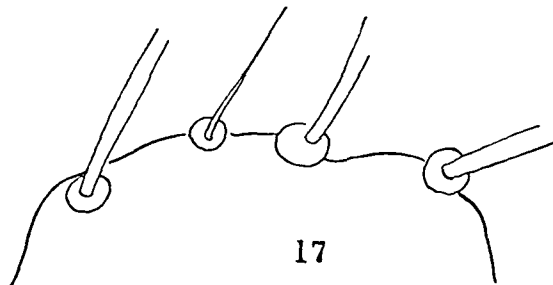
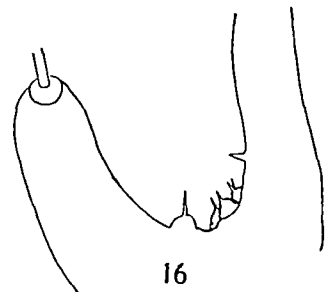
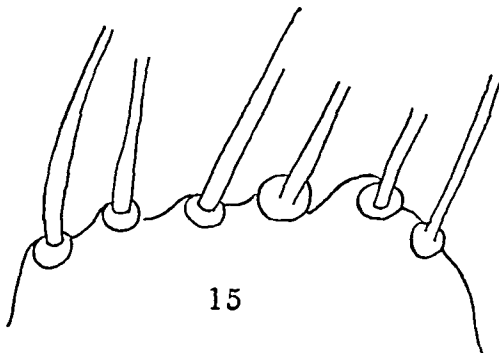
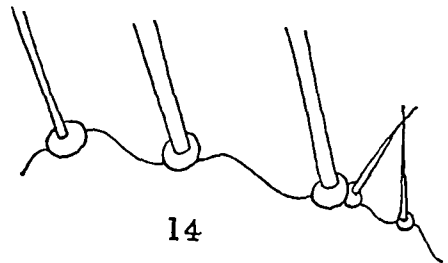
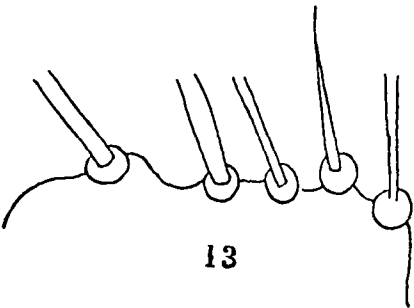
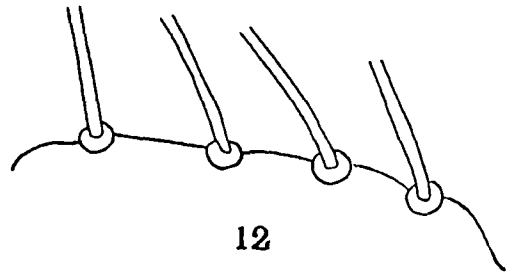
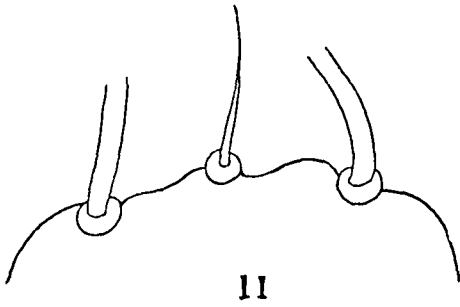
PLATE XXXI.



Figs. 1 to 10.— Posterior part of the paramere of one side, showing the variation in the position and length of the spines in *C. megacephala*.

del. Roy.

PLATE XXXII.



Figs. 11 to 15.—Showing the number of hairs on the ventral edge of anterior paramere in *C. megacephala*.

Fig. 16.—Showing the posterior part of the paramere in *C. bezziana*.

„ 17.—Showing the number of hairs on the ventral edge of anterior paramere in *C. bezziana*.  
del. Roy.

## NOTES ON ANTIRABIC IMMUNIZATION: A CORRECTION.

BY

LIEUT.-COLONEL G. COVELL, M.D., D.P.H., D.T.M. & H., I.M.S.,  
*Late Officiating Director, Pasteur Institute of India, Kasauli.*

[Received for publication, December 12, 1936.]

A PAPER entitled 'Notes on Antirabic Immunization' by Covell, McGuire, Stephens and Lahiri appeared in the *Ind. Jour. Med. Res.*, **24**, 2, pp. 373-388. In it was given an account of certain experiments which were carried out or completed during the two years in which the present writer was officiating as Director of the Pasteur Institute of India.

At the beginning of Section 1, which dealt with the use of antirabic serum and live fixed virus as adjuncts to treatment with carbolized vaccine, the following sentence occurred:—

'The results of previous experiments in this connection were reported by Shortt *et al.* (1935). Those now recorded represent a continuation of the same investigations'.

The reference was to a paper by Shortt, McGuire, Brooks and Stephens published in the same *Journal*, **22**, 3, pp. 537-556.

A similar sentence should have been inserted at the head of Sections 5, 6, and 7, which gave an account of experiments in connection with the keeping properties of carbolized vaccine, the relationship between complement-fixation tests and immunity to rabies, and the immunizing value of a rabies virus fixed in the dog.

These experiments were all initiated by Lieut.-Colonel Shortt, I.M.S., to whom I wish to make my acknowledgments.



## NOTICE.

*The following has been received for announcement :—*

*—Editor.*

### BUREAU OF HUMAN HEREDITY.

115, Gower Street, London, W.C.1, England.

THE object of this Bureau is collection on as wide a scale as possible of material dealing with human Genetics. Later, the tasks of analysis of material and distribution of the information available will be added.

The Bureau is directed by a Council representing medical and scientific bodies in Great Britain. It is affiliated to the International Human Heredity Committee, which ensures co-operation in all areas where research is proceeding.

The Council would be grateful to receive all available material from Institutions and individuals, furnishing well-authenticated data on the transmission of human traits whatever these may be. Pedigrees are particularly desired; twin studies and statistical researches are also relevant. As research workers and others who send in material may in some cases wish to retain the sole right of publication (or copyright) those who so desire are asked to accompany their material with a statement to that effect.

Material should be given with all available details in regard to source, diagnostic symptoms, and the name and address of the person or persons who vouch for accuracy. All such details will be regarded as strictly confidential.

Reprints of published work would be most acceptable. Further, many authors when publishing material may also have collected a number of pedigrees which they have been unable to reproduce in detail. It is the object of the Council that such records, by being included in the Clearing House, should not be lost.

Those wishing for a copy of the Standard International Pedigree Symbols may obtain one from the office.

Announcements in regard to the services undertaken by the Bureau will be published from time to time.

*Chairman* : R. Ruggles Gates.

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C. B. S. Hodson (Hon. Gen. Secretary).



## NOTICE.

### **Fourth Course of Post-graduate Instruction in Malariology, under the auspices of the League of Nations, Singapore.**

*April, 1937.*

THE League of Nations is arranging for a fourth Course of Instruction in Malariology which will commence at the King Edward VII College of Medicine at Singapore, on Monday, the 19th April, 1937.

The object of the Course is to complete the training of medical practitioners who are engaged, or intend to be engaged, in the work of the malaria control in their own countries. The Course will thus be of interest not only to Governments and Municipal authorities, but also to all medical men practising in Eastern countries, particularly those engaged in estate work.

The Course will comprise two stages, one consisting of theoretical, clinical, and laboratory studies with practical demonstrations, and the other devoted entirely to practical field studies.

The first stage will commence on 19th April and continue until the end of May. It will be carried out partly at the King Edward VII College of Medicine and partly at the Tan Tock Seng Hospital, Singapore.

The second stage will commence at the beginning of June, when candidates will proceed in groups either to Indo-China, the Netherlands Indies, or Malaya, where they will have the opportunity to study the routine work of malariologists and the application of control measures under field conditions. This stage of the Course will last 14 to 21 days.

*Conditions of admission.*—The Course is open to medical practitioners, from whom applications for admission will be received up to the 27th February, 1937.

*Fee.*—The fee for the whole or any part of it is Seventy-five Straits Dollars (\$75), payable in advance.

*Fellowships.*—The League of Nations is making available a limited number of Fellowships which will only be granted to candidates nominated by their Governments or semi-official institutions.

*General.*—Further information concerning the conditions governing the granting of fellowships or admission to the Course will be supplied on application to the Director, League of Nations Eastern Bureau, 336 River Valley Road, Singapore.

—Editor.

## SYLLABUS.

THEORETICAL, CLINICAL AND LABORATORY STUDIES, WITH  
PRACTICAL DEMONSTRATIONS.**1. Entomology :—**

This section of the Course will comprise lectures and practical laboratory work spread over a period of four weeks and lasting for approximately 58 hours.

*Syllabus.*—The detailed morphology of anopheline imagines, pupæ, larvæ, and ova. The œcology of imagines, larvæ, and ova; physical and biotic autœcology, synœcology. The genus *Anopheles*, its classification and affinities; the species and categories below the species; geographical and seasonal distribution; the characteristics of the principal carriers of the world. Susceptibility; influences determining the efficiency of carriers. The determination of species in all stages; the use of keys and original descriptions in a study of the Eastern fauna. The technique of laboratory and field investigations.

**2. Haematology and Protozoology :—**

A series of lectures and laboratory classes comprising ten lectures and seventeen hours' practical laboratory work.

*Syllabus.*—Blood cytology, staining methods, enumeration methods. General consideration of the protozoa with particular reference to the sporozoa (plasmodium, hæmoproteus, etc.). Detailed study of the malarial parasites. Malaria in birds, monkeys, and other animals. (A more detailed syllabus will be available before the Course begins.)

**3. Pathology :—**

Immunity under experimental conditions. A series of lectures, demonstrations, and laboratory studies lasting ten and a half hours.

*Syllabus.*—Nature of the malarial paroxysm. Pathogenicity of malaria parasites—virulence of parasite and susceptibility of host. Changes in organs and tissues in acute pernicious, in benign, in chronic and latent malarial infections, and in super-infections—correlation with clinical syndromes. Toxins, changes in the blood; cellular, biochemical and immunological. Immunity—natural immunity and acquired immunity (resistance, tolerance, premunition). Cellular basis for immunity. Antibodies. Factors influencing immunity—therapy, splenectomy, blockade, etc. Duration of immunity. Relapse. Spleen size and parasite prevalence in relation to immunity. Infection and super-infection under experimental conditions. Strains of malaria parasites—specificity; importance in relation to specific therapy and epidemiology.

**4. Clinical :—**

A series of lectures and clinical demonstrations extending over a period of four days.

*Syllabus.*—Demonstration of selected cases and discussion. Relapses and resistance to malaria; provocation of attacks. Induced malaria; delayed manifestations; complications and sequelæ. Toxicology in relation to the use of quinine and other drugs. Determination of what constitutes a cure. Blackwater fever.

**5. Therapeutics :—**

A series of six lectures of one hour each.

*Syllabus.*—Plasmoquine, Fournau 710, Atebrin, arsenicals, and other preparations. Pharmacology and toxicology; dosage and administration. Therapeutic effects; effects on the various stages of the parasites; effects on relapses. The present status of synthetic drugs in comparison with quinine and its derivatives.

**6. Epidemiology :—**

A series of 12 lectures of one hour each.

*Syllabus.*—Methods and interpretation of results; measurement of malaria (spleen rate, parasite rate, sporozoite rate, etc.) and the estimation of its prevalence and intensity; statistical analysis of



data. Spleen—theory. Endemicity, epidemicity, and pandemicity, their characteristics and the factors involved. The prediction of epidemics. Topographical, climatic, and social factors. Anopheles *sine malaria*.

### 7. Control :—

A series of lectures and lecture-demonstrations will be given occupying a minimum period of 60 hours. Field demonstrations on urban malaria control will be arranged through the courtesy of the Municipal Health Officer, Singapore. Opportunity will be given to students to familiarize themselves with field conditions in association with the theoretical lecture-courses in Entomology and Epidemiology.

*Syllabus.*—The history of malaria control in Singapore. The organization of anti-malaria services in urban and rural areas. The classification and application of malaria-control measures. The importance of drainage. Species control and the choice of methods suitable to a particular locality. The influence of biological knowledge on the control of malaria. The limitations and uses of larvicides. Malaria surveys; the measurement of malaria in the field. The relative cost of malarial endemicity. Temporary (recurrent) and permanent control measures, their efficiency, cost, and financial benefit. Anti-malaria legislation and propaganda.

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## A STUDY OF THE VIBRIO GROUP AND ITS RELATION TO CHOLERA.

BY

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S. R. PANDIT, D.B., M.B., B.S.SC.,

*Ind. Jour. Med. Res.*, **24**, 4, April, 1937.

### CORRIGENDUM.

In paper entitled 'Adsorption of Antigens by Antibodies or vice versa. Part III' by B. N. Ghosh and N. N. Ray, *Ind. Jour. Med. Res.*, **24**, 3, January, 1937, p. 629, Table IV, for 'tetanus' read 'diphtheria'.

Mackie and Storer (1918) working in Egypt isolated from cases of choleraic diarrhoea vibrios inagglutinable with cholera high-titre sera, to which they gave the name 'paracholera' vibrios and concluded that 'it is probable that this group comprises a number of different types and species' and that the 'evidence is strongly in favour of these strains being causally related to the cholera-like disease with which they were associated'.

Similarly, Greig (1916) investigated a series from Calcutta water sources and from 31 vibrios defined six serological groups.

The serological examinations made by these and other workers were however not specifically directed to an examination of the heat stable ('O') antigen, although the method of examination probably resulted in readings which would indicate it (Gardner and Venkatraman, 1935). The value of the separate estimation of 'O' agglutination in the course of the examination of vibrios was shown in the reports of certain workers, notably Bruce White, in the course of the trials which had been carried out with sera prepared in connection with an investigation directed towards the production of a standard cholera agglutinating serum, and which are embodied

in the collected series of reports published by the Office International d'Hygiene Publique (1934).

The first detailed study of vibrios of a variety of sources of origin, including strains other than typical *V. cholerae*, was carried out by Gardner and Venkatraman (*loc. cit.*) who, on the results of examination of a series of 47 strains other than the classical type, defined five 'O' serological groups in addition to the typical *V. cholerae*, 20 strains being classified in the five groups and 27 being serologically individual.

The present paper is based on an investigation into the characters of vibrios carried out as part of a larger scheme with which other cholera inquiries which have been established in India were associated. At Kasauli a detailed study of the serological and biochemical characters of recently isolated strains was undertaken. The object of this portion of the Inquiry was the examination of strains isolated from cases of clinical cholera, healthy carriers and water from endemic and non-endemic areas in India, for the purpose of ascertaining whether vibrios of other than the classical type and of any consistent nature could be ætiologically associated with the disease, the distribution of such strains in different areas and in different sources and their possible relationship to the classical type. It was intended that this should form a basis for future bacteriological and epidemiological studies in India, both in endemic and non-endemic areas. No attempt has been made to secure results further than those obtainable by simple agglutination tests and investigation of biochemical and morphological characters.

#### SOURCE OF SUPPLY.

The main source of strains for examination was the endemic area of Bengal. There had been established in Calcutta an Inquiry at the Campbell Hospital, in which special wards exist for the treatment of cholera, directed chiefly towards tests of the therapeutic value of bacteriophage, and in the course of the work all cases clinically resembling cholera which were admitted were submitted to routine bacteriological examination. From this source a large number of freshly isolated strains was available which included many inagglutinable strains. (The term 'inagglutinable' is applied in this communication to vibrios, which do not show the 'O' antigen of the typical *V. cholerae*.)

In the vicinity of Calcutta an area had also been selected for an Inquiry into the carrier problem and in this area with a population of about 10,000 routine systematic examination of healthy individuals was in progress. A large number of stools and water sources was examined and from these inagglutinable strains were also obtained. This area may be considered a typical endemic area.

Strains were also obtained from certain other areas.

Table XI shows that the chief sources other than Bengal are Mandapam, a quarantine station in the south of India through which emigrant labourers pass from Madras Presidency to Ceylon, and Rajputana, a dry hot area, where the incidence of cholera is in most years low, from which a series of water vibrios were collected by the authors. The latter sources consisted of jhils or lakes, rain-water ponds, wells in ordinary use, rivers, drains, taps, etc. The table shows sufficiently clearly the origin of the remainder of the strains.

In all a total of 558 vibrios has been dealt with. Of these 354 were isolated in the Bengal area and 204 from other sources. Two hundred and two of these were isolated from cases of clinical cholera, 182 from healthy persons and in occasional cases from those suffering from other diseases, and 172 from water sources. An approximately equal number of vibrios from the three main sources is therefore dealt with. Unfortunately the material available does not bear the same proportions in the endemic and non-endemic areas. A majority of case and carrier strains is examined from Bengal and a majority of water strains from other sources. The material isolated in Bengal in the epidemic year 1935-36 is recorded separately as there is available evidence of the epidemiological conditions prevailing during that year. The latter are considered in a separate paper.

In this investigation, although at least 1,000 different strains have been examined, only 558 are reported as, where biochemical and serological investigations have proved similarity between strains from the same source, whether case, carrier or water, duplicates have been excluded. With regard to case strains this means that the series of case vibrios from all sources, numbering 202, contains four examples in which two strains of different characters from one patient are included, one example in which three strains and one example in which four strains from one patient are included. The remainder represents one vibrio per patient. The position with regard to carrier strains is similar as there are very few strains of different types from the same individual. In the case of the water strains the position is different. Many strains from the same source are unclassified and therefore their serological character has not been worked out; amongst these it is probable that duplicates from the same source have been included. Amongst these, however, where serology and biochemical reactions have shown similarity in the one source, even though isolated at quite different periods, duplicates have been excluded.

#### SEROLOGICAL INVESTIGATION.

The strains submitted to serological investigation were those which did not agglutinate with a serum prepared against dried 'O' antigens of Inaba and Ogawa types issued from the Standards Laboratory, Oxford, for experimental trial. The further serological testing of these strains was carried out by means of 33 sera whose characters are given in Table I. These include:—

- (a) Type strain 'O' groups, Gardner and Venkatraman, II to VI—5 strains.
- (b) Selected non-agglutinating strains from cases of clinical cholera isolated in Calcutta—13 strains.
- (c) Selected case strains from other sources—8 strains.
- (d) Selected strains from Bengal carriers—4 strains.
- (e) Selected strains from Bengal water—2 strains.
- (f) Kohat water strain—1 strain.

In addition a serum was prepared against the rough variant of Inaba (Bruce White, 1935) and was used as a check on all vibrios. Any vibrios agglutinating with this serum have been excluded.

The technique employed is given in the Appendix.

Table II shows the results obtained. It will be seen that a number of definite serological groups has been obtained. The most striking fact, however, that emerges from a consideration of the tables is that, while 558 strains are listed, the largest serological group contains 40 vibrios only. There are two other groups of 40 and 30, two of 17, two of 16, one of 12, three of 11 and three of 10 and the remainder of the 31 groups are in single figures. In addition four of the sera used have agglutinated the homologous strains only. There is a certain amount of overlapping amongst the groups but each vibrio has been included in one group only. The table shows the strains Calcutta carrier 2898 and Calcutta case 324/7 in one group, as these were only different in having their own type specific antigen with a common group antigen. Shillong case strain 984 and Gardner's group IV are also included together as, although biochemically different, they are serologically very similar and agglutinate the same strains. In addition there was considerable relationship between Calcutta carrier 2943, Bengal water 1A, Calcutta case 1805/1, while Shillong case strain 3067 and Lahore case 10/5 have some points in common. Thus, although the grouping might be arranged to show one more large group serologically similar, the general finding is in any case the same that, in spite of the use of 33 sera, a maximum of four large groups has been obtained in 558 vibrios and the maximum percentage incidence of these groups is 7.2 per cent, while 247 vibrios remain unclassified.

The percentage incidence of the groups in the various sources is given in Table III. In view of the smallness of many of the groups the value of this table is diminished, but it shows that vibrios of even the smaller groups can be detected in more than one of the different sources, in spite of the very low percentage incidence that these groups may occupy on the whole. There is no group containing more than four vibrios that has not been found in at least two of the sources, while speaking generally those vibrios which are well represented in one source are also represented in at least one of the others. This is true not only for the vibrios as a whole, but also for the vibrios whose isolation dates and circumstances of isolation are known, as represented by the material collected in Bengal in the epidemic year 1935-36. The latter figures are also valuable in showing that there is no one particular period in which any one type is especially prevalent with the exception of 'Rangoon Rough'. In other words, no series of isolations of any size was due to any vibrio of one particular type.

Rangoon Rough represents 22.3 per cent of the inagglutinable vibrios isolated in the epidemic period, far the largest percentage incidence attained in the series. It has been isolated from cases in other areas than Bengal. It has been found on the same plate with the agglutinable vibrio on two authentic occasions. It has been isolated from at least three carriers and the classical Metchnikovi strain belongs to this serological type and is identical in its biochemical characters.

The percentage of vibrios classified is largest in 'cases' and less in 'carriers' and water and this applies also to the vibrios collected outside Bengal. Only five of the sera were prepared against carrier strains and of these two agglutinate only the homologous organisms: four of the sera were prepared against water strains. The remainder of the sera was against case strains. Two of the carrier sera agglutinate 17 and 11 vibrios respectively and that agglutinating 17 vibrios agglutinates two case strains only, while that agglutinating 11 does not agglutinate any case vibrio. One of the three water vibrio sera agglutinates no case vibrio

(group of 8) while the others agglutinate two and four out of a total of 12 and 11 respectively in the group. The proportion of case, carrier and water strains agglutinated by case vibrio sera is given in Table IIa. As the total number of case, carrier and water strains dealt with is approximately equal, it is clear from these facts that case sera have a closer relationship to case vibrios, while carrier and water sera have a lesser relationship to these vibrios.

From what has been said as to the method of selection of strains for inclusion in this report it will be clear that the percentage of strains classified is the minimum possible with the material received and the minimum claim is therefore put forward as to the classification obtainable. On the other hand, the classification is a purely serological one and some of the serological groups contain strains which give different biochemical reactions. The extent to which this has occurred can be gathered from Table IV, the findings of which will be commented on later. In addition, vibrios showing only 25 per cent of titre have also been included but only after a careful check of the findings. As the groups are not completely homogeneous it has not been thought worth while to separate these latter off, especially as their total number in the entire series is only 14 vibrios. It will be noted that from the 139 inagglutinable vibrio cases admitted to the Campbell Hospital in the epidemic year 1935-36, 112 vibrios have been investigated, representing 107 patients.

#### BIOCHEMICAL REACTIONS.

(a) *Fermentation tests.*—The fermentation reactions of all strains in this series were tested on mannose, saccharose and arabinose, the sugars which Heiberg (1935, 1936) has shown to be of special differential value in the examination of vibrios. In addition, glucose, lactose and mannite were also employed. The results of previous examinations of 494 vibrio strains from different sources, including both agglutinable and inagglutinable strains, had been reported (Taylor, Read and Pandit, 1936) and the finding of Heiberg that all agglutinable strains produced acid from mannose and saccharose but not from arabinose had been confirmed. In the examination of the present series of 558 strains which have also been submitted to serological examination, a classification of vibrios into the six fermentation types of Heiberg was carried out. One strain has been found of a seventh fermentation type. Taking the results with glucose and mannite, in addition to those with the three other sugars, it has been considered advisable to divide Heiberg type VI into two sub-types: sub-type 1, in which no sugar including glucose is fermented, and sub-type 2, in which glucose and mannite are fermented but not mannose, saccharose and arabinose. Strains belonging to these two sub-types, which differ in other biochemical reactions as well, would not have been differentiated if only the three essential sugars had been employed. Similarly within the types I and V, there were 11 and 3 strains respectively that did not ferment mannite; these have been separated and have hereafter been called aberrant types I and V. Nine out of the 11 aberrant type I strains and all the 3 strains of aberrant type V were Rajputana water strains. All the 10 strains of type VI, sub-type 1, were also water strains. Lactose was not fermented in 24 hours by any of the strains of the whole series except by two water strains. Glucose was fermented by all the strains except those of type VI, sub-type 1, and mannite was fermented by all except by type VI, sub-type 1, and the aberrant types I and V strains.

The results of fermentation tests are given in Table VII. The majority of the strains (61 per cent) gave the reactions of Heiberg type II. The next big group (18 per cent), as in our previous report, gave the reactions of type I, which are also given by all agglutinable strains. All the other types together accounted for 21 per cent of the total inagglutinable vibrios.

(b) *Cholera-red and indol reactions.*—These reactions were tested on all strains and it was found necessary, as is mentioned in the Appendix, to check very carefully the suitability of the sample of peptone used for test.

Ninety per cent of strains of fermentation type I gave a C-R+ reaction: out of 342 strains of type II, only one gave a negative reaction. The strains of types III, IV, and V gave a negative reaction, while in type VI a negative reaction was given by sub-type 1 and a positive by sub-type 2. The results were variable with strains which did not ferment mannite (Table VIII). Shortly, it can be stated that, with few exceptions, vibrios giving fermentations of types I, II, and VI, sub-type 2, and the non-mannite fermenters (aberrant types I and V) were cholera-red positive and those of fermentation types III, IV, V, and VI, sub-type 1, were without exception cholera-red negative. The type VII strain is also cholera-red negative.

The indol reaction was positive with all C-R+ strains. In the case of strains giving a C-R— reaction, results were variable. The irregularity of the test does not make it of much value, but it is noted that all the cholera-red negative strains of type V were indol producers.

(c) *Voges-Proskauer reaction.*—Typical *V. cholerae* gives a negative V-P reaction. This reaction has been employed in the examination of our atypical strains. The modification introduced by Barritt (1936) was used in which  $\alpha$ -naphthol is employed along with potassium hydroxide, the test so modified being much more sensitive than the ordinary one. Both the modified methods and the ordinary V-P procedure (see Appendix) were carried out on 351 inagglutinable strains, 90 non-hæmolytic agglutinable strains and six hæmolytic agglutinable (El Tor type) strains. In many cases a positive result was obtained with Barritt's modification where the ordinary test was negative, but the reverse was not found. Thus, out of a total of over 280 positives by the modified method, 32 were negative and 16 doubtful by the ordinary test.

The agglutinable non-hæmolytic vibrios tested, which were mostly of the Inaba type but also included several Ogawa and 'middle' types and also the Inaba rough derivative, were all negative by the modified test. All of them were cholera-red positive.

An interesting relationship is found between the cholera-red and the modified V-P tests, especially when these are considered along with the results of fermentation tests. Table IX shows the results with *inagglutinable* vibrios. It will be seen that there is a close parallelism between the modified V-P test and the cholera-red reaction. In a total of 351, the V-P test is positive where the cholera-red reaction is positive and negative where the cholera-red is negative, the number of exceptions being 23. If the strains of the aberrant types I and V are omitted there are 13 exceptions as against 326 which conform to the above statement. The inagglutinable vibrios fall into two main groups on the basis of the combination of their reactions with C-R and V-P tests. The larger group in our series is C-R+V-P+, which contains 240 out of a total of 351. In this group, strains of Heiberg types I



and II form the great majority, the exceptions being shown in the table. The smaller group gives the reactions C-R—V-P—. These are strains belonging to Heiberg types III, IV, V, VI-1, and VII with few giving aberrant reactions. Of the five strains of type VI-2, three human strains are in the former larger group, the remaining two water strains being exceptions (C-R+V-P—).

Mention has already been made that agglutinable non-hæmolytic vibrios tested gave the reaction C-R+V-P—. In the series of 351 inagglutinable strains examined, only 15 gave the same results and of these 10 were of types aberrant in their sugar reactions from the recognized Heiberg types. No inagglutinable strain of Heiberg type I has given the reactions C-R+V-P—. It is therefore possible, on biochemical evidence alone, to obtain presumptive diagnosis of the serology of the typical *V. cholerae*; if it gives the fermentation reactions of Heiberg type I, is cholera-red positive and negative to the modified V-P test, it is very probably an agglutinable vibrio. Just as the results of fermentation and cholera-red tests are available 24 hours after the isolation of a vibrio, so too is the result of the modified V-P test. The test performed on a small number of strains gave a slightly weaker, but still a marked reaction, in 24 hours than in a three-day culture; it is, however, necessary to inoculate the glucose phosphate broth rather liberally. The fermentation, cholera-red and modified V-P tests can therefore be profitably performed along with the agglutination test and read with it.

Six strains of the El Tor type (hæmolytic, agglutinable) in our collection have also been tested. Five gave a positive reaction to the modified V-P test and the sixth was negative. The number tested is too small to justify any conclusion but it may be said that, in respect of this test, the El Tor type is similar to the inagglutinable vibrio in being C-R+V-P+.

(d) *Test for hæmolysis*.—The hæmolysis of all strains was tested on goat cells by a method (see Appendix) similar to that introduced by Greig (1914). No agglutinable vibrio amongst our collection of Indian strains was found to be hæmolytic and it is of interest that, in an examination of 333 typical *V. cholerae* strains, Greig did not find any agglutinable hæmolytic strain, the type to which the name 'El Tor' has been given.

The results of hæmolysis tests of inagglutinable strains from cases of clinical cholera, carrier and water were very variable and no relationship was found to the source of origin or other biochemical reactions. In some cases, after varying periods of subculture and plating out, differences in the results were noted with strains.

(e) *Gelatin liquefaction*.—The test for gelatin liquefaction has not been carried out as a routine on all the strains but only on such strains where the species of the organism was in doubt and later on some of the strains representing each of the different Heiberg fermentation types. Table X gives the results of this test. It may be mentioned that, so far as the strains examined go, all the fermentation types, including the aberrant types, liquefy gelatin with the exception of types IV and VI, sub-type 1, in which some liquefy and others do not liquefy gelatin. The great majority of strains of type VI, sub-type 1, do not liquefy gelatin. Gelatin liquefaction is not related to any of the other biochemical tests carried out; thus 81 C-R+ and 87 C-R— strains liquefied gelatin and the 13 non-gelatin liquefiers

were all C-R—. The test is therefore of little differential value, but probably non-gelatin liquefiers are found only among C-R— strains.

#### RELATION OF SEROLOGY TO BIOCHEMICAL REACTIONS.

Tables IV and V show the degree of homogeneity obtained in each of the groups by reference to their sugar reactions according to the classification of Heiberg (1935). (The authors have added certain new types that have occurred in their series.) It will be seen that by no means all vibrios of one serological group are identical in their sugar reactions. A total of 53 exceptions out of 311 classified is recorded. Of these seven strains show a 25 per cent reaction only with the serum, i.e., half of the vibrios in this series that show partial titre (below 50 per cent).

In the section on biochemical reactions it has been shown that there is reason to group together organisms giving a positive reaction with both cholera-red and modified V-P tests. The degree of variation from normal amongst the serological groups on this basis can be seen in Table VI. In all 17 vibrios only show differences. Of these 10 are to be found amongst the Rajputana water strains, three showing a 25 per cent reaction only and four have the cholera-red reaction positive but the modified V-P negative: three of the exceptions are the strains from which the serum was prepared, while one exception is an organism auto-agglutinable in 0.85 per cent saline.

Another point worthy of notice is that of cholera-red positive case vibrios 86 per cent have been classified and of the cholera-red negative 24 per cent only. The figures for carriers are 56 per cent and 10 per cent, for water strains 46 per cent and 25 per cent, and for all strains 64 per cent and 23 per cent. Nearly three times as many vibrios in the former group are classified as in the latter. Of the sera used seven include agglutinins against strains of cholera-red negative strains. Of these six are negative to the modified V-P test. These agglutinated in all 16 strains besides the homologous strains of which six only are cholera-red negative and of these four are agglutinated by one serum and include two vibrios isolated from the same area as the strain against which the serum was prepared. Four of the sera agglutinate no vibrio of this type except the homologous strain. Two cholera-red negative Calcutta strains from a carrier and water, respectively, were used to prepare 'H' and 'O' sera. These sera have agglutinated no other strains than the homologous. A third point is that the 'H' and 'O' Inaba sera (titre usually 2,500) used by the authors have agglutinated none of the cholera-red negative strains, while they have agglutinated 38 per cent of C-R positive strains. With one particular experimental serum prepared against a chemically extracted Inaba fraction, which was mainly of protein nature but contained a small residue of the polysaccharide, results of special interest were obtained. The exact nature of the serum raised by means of this fraction is uncertain and it has not been possible to proceed further up to the present with a study of this serum; its use however has shown possible relationships between vibrios not obtainable by other means. The findings with this serum will be given, but with reservations as to the exact nature of the factors concerned in agglutination tests. This serum agglutinated to varying titres 93 per cent of cholera-red positive strains (total 98) and failed to agglutinate 97 per cent of cholera-red negative strains (60 strains). The only C-R— organisms

that agglutinated were Kohat Original and Kasauli 11 (Heiberg V organisms). While it is true that the majority of the sera used was prepared against cholera-red positive organisms, it is also true that the sera prepared against the cholera-red negative organisms agglutinate a minority of cholera-red positive strains. At the same time 10 cholera-red negative strains have reacted with cholera-red positive sera, six of which are included amongst the Rajputana water strains. It may be concluded therefore that the cholera-red negative strains, both from an agglutinogenic and agglutinable standpoint, are more individual than the cholera-red positive; in other words, the degree of heterogeneity is greater amongst them. In any attempt at grouping vibrios on the basis of the combined action of cholera-red and V-P tests it must be recognized that vibrios negative to these tests are not to be regarded as similar on any other count. The cholera-red positive organisms have that reaction in common with the agglutinable vibrio, though they differ in their reaction to the modified V-P test: they also, as pointed out above, react with two sera made against the classical agglutinable vibrio, while against certain of the inagglutinable vibrio sera they behave similarly, in spite of differences in their Heiberg sugar reactions.

#### RELATION OF SEROLOGICAL GROUPS TO GEOGRAPHICAL SOURCE.

Table XI shows the serological groups according to geographical source. Excluding those that contain the homologous strains only four groups are found in one geographical source only and these contain 12, 10, 4, and 3 vibrios, respectively.

#### VARIATION OF STRAINS.

In the course of this investigation no example of variation amongst the strains has been experienced in the course of ordinary subculture. Strains were however stored in a manner that probably provides conditions in which variation is unlikely to occur (*see Appendix*).

Certain difficulties have been experienced in securing highly agglutinable emulsions, but these have been overcome by the laboratory procedures detailed in the section on 'Technique'.

The use of the Inaba rough serum has enabled variants of this type in cases to be detected, the strains being sent to Kasauli as inagglutinable vibrios and being found on examination to possess varying degrees of titre with the Inaba smooth and rough sera. These vibrios represent the only examples of partial titre reactions (i.e., under 50 per cent) with the Inaba smooth serum. All strains of the typical smooth agglutinable type reacted to full titre. Moreover, the strains originally isolated as partial roughs in the course of subculture eventually became perfectly smooth. By colonial selection it was possible to obtain strains that reacted to 100 per cent with the rough or the smooth sera respectively, showing them to be mixed strains. The experimentally derived Inaba rough, on the other hand, has always remained stable. This strain has been compared with one supplied by Bruce White and is identical. The Kasauli strain was derived by Bruce White's (*loc. cit.*) method. This remains, therefore, the only example of variation in nature that has been discovered during the course of this investigation and the only

serological type which can be definitely stated to be related to typical *V. cholerae* so far as our observations have gone.

#### MULTIPLICITY OF TYPES IN THE SAME CASE.

The following examples of this occurrence are recorded :—

1. Rangoon Rough isolated on the same plate as a typical agglutinable vibrio—on two occasions in different geographical areas.
2. Lahore case 9—on the third day of the disease Rangoon Rough and an agglutinable vibrio were isolated ; on the fourth day type strain Lahore case 9 and an agglutinable vibrio were found.
3. Calcutta case 2148—a Campbell Hospital case—studied in detail at very short intervals gave the following results :—

Serum positive.			
1st specimen	..	..	Gardner's III.
2nd	"	..	} Madras case 10.
3rd	"	..	
4th	"	..	
5th	"	..	
6th	"	..	
7th	"	..	} Calcutta case 324/7.
3rd	"	also	

4. Calcutta case 1499/1—a Campbell Hospital case. This strain was examined in Kasauli and filed. After several weeks it was found to consist of two vibrios with Heiberg II and Heiberg III sugar reactions, the former agglutinated to titre with Calcutta case 1612 serum while the latter agglutinated with a Lahore case serum.

In this connection it may be mentioned that file copies are put up immediately on receipt of the specimen, also in the examination of the Rajputana water strains it was found on many occasions that several platings were necessary to ensure the purity of a strain. It is quite certain that it is possible to obtain vibrios of two types from a single colony even over three successive platings.

5. Calcutta case 627—a Campbell Hospital case. On the first day of examination an inagglutinable vibrio was isolated that reacted with none of the sera ; on the second day type strain Calcutta case 627/2 (Heiberg V) ; on the third day Calcutta case 627/3 (Heiberg II) belonging to the common group represented by Calcutta carrier 2898 and Calcutta case 324/7.

6. Lahore case 10. The stools of this case were received in a sealed peptone water capsule and plated 24 hours after collection. Three vibrios were isolated—Lahore case 10/6<sub>T</sub> (Heiberg III) which reacts with no sera, Lahore case 10/6D<sub>0</sub>

(Heiberg I) which reacts with no sera, and Lahore 10/6D<sub>2</sub> (Heiberg I) which reacts with serum Calcutta carrier 2943.

Such findings could be multiplied and in the Rajputana water series there were many examples.

It seems reasonable to regard these findings as serving to accentuate the multiplicity of types that exist in nature, so that if infection occurs from external sources more than one of the types present in that source are likely to be found in the stools.

#### DISCUSSION.

The serological and biochemical examination of this large series of recently isolated vibrio strains has shown the extreme heterogeneity of the group. On the basis of simple agglutination tests without a detailed antigenic analysis 56 per cent only of the strains have been classified. In order to effect this, sera were prepared against 33 vibrio strains and 31 serological groups have been defined. Of the 311 vibrios classified, 57 were classified in Gardner and Venkatraman's groups II to VI, 40 being classified in their group III. The remainder belong to newly defined serological groups. It is clear that a very large number of sera would be necessary for any complete classification and even then it is unlikely that a series of the size dealt with here includes more than a fraction of the existing vibrio types. It is shown that the serological heterogeneity of the cholera-red negative strains is of a very much higher degree than of the cholera-red positive organisms.

On the basis of biochemical reactions a similar degree of heterogeneity was demonstrated. By a combination of the reactions with six sugars, the cholera-red test and modified Voges-Proskauer tests inagglutinable vibrios of seventeen different types have been distinguished. Of these thirteen have been isolated from cases of 'clinical' cholera.

This heterogeneity moreover is exhibited by vibrios from all the three sources, both in endemic and non-endemic areas—vibrios of even the smaller serological and biochemical types being found in most cases in more than one of the sources. It is a striking fact that vibrios identical on all counts can be isolated from all the three sources, this positive evidence being of greater importance than that derived from a failure to isolate in certain instances from more than one source.

Not only is there this similarity of findings in cases, carriers and water, but when geographical sources of origin are considered it is found that these vibrio types are widespread and may exist in sources in which the possibility of recent contamination from a cholera case is extremely unlikely. Thus seven of the Rajputana water vibrios differ in no way from case vibrios obtained in other parts of India and China on the basis of simple agglutination tests and biochemical reactions, and if sugar reactions be excluded the number of Rajputana vibrios, identical serologically and in their cholera-red and Voges-Proskauer reactions with such vibrio, is eighteen. Two strains received from Batavia in the absence of cholera in that locality, one from a case of diabetes and the other from a case of typhoid fever, reacted with a Calcutta case strain serum and Gardner's group VI. A Lahore case strain has agglutinated a contaminant strain obtained in the course of mouse passage in Kasauli, though the vibrio was of different biochemical type.

The 'Kohat Original' strain isolated from water in 1934 in the North-West Frontier Province in the absence of any recorded case of cholera in the entire province has agglutinated Calcutta carrier, and Bengal and Rajputana water strains, though their biochemical reactions differ. A Calcutta case strain is identical with two old case strains isolated in Basrah, and obtained by this Institute from Roumania. The Rangoon Rough serum has agglutinated a strain of *V. metchnikovi* isolated in Odessa and sent from Hamburg, and is identical with it. It is true that very few absorption tests have been carried out, but the similarity of the majority of the above vibrios is not in question.

The findings of the present work show that the results of serology and the cholera-red test are, with extremely few exceptions, uniform. The Voges-Proskauer test to a marked extent follows the cholera-red test. The results of fermentation tests show finer differences. It is noticeable that serological results do not in many cases distinguish these differences. In the case of the agglutinable vibrio, with the sole exception of the El Tor strains, the results of all examinations carried out by the authors are uniform. With the inagglutinable vibrio strains it appears that an almost infinite variety of combinations is possible, although the vibrios within one serological group are for the most part uniform on all counts. This is merely an expression of the high degree of heterogeneity of inagglutinable vibrio strains, as opposed to the almost complete uniformity presented by the agglutinable vibrio strains.

In this series in the case of only one group is there any evidence of possible causal relationship with cholera. The 'Rangoon Rough' strain showed a relatively high incidence during the epidemic year 1935-36, eighteen vibrios of this type being isolated over a period of two months during the epidemic period. At the same time other inagglutinable vibrios were also being isolated and hundreds of cases were occurring in which the agglutinable vibrio was found. This vibrio has twice been found on the same plate as the agglutinable vibrio. It has also been isolated from three carriers. In no other case has any sequence of isolations of the same serological type other than the classical from *different* cases or other sources occurred in this series.

It has, however, to be admitted that although the number of vibrios from each source examined is almost the same, the sera most of which are prepared against case vibrios have agglutinated a higher percentage of case strains, while the reverse is the case with sera prepared against carrier and water strains. It is true that if inagglutinable case, carrier and water vibrios were of the same origin, with a sufficiently large series to ensure true random sampling it would have been expected that the percentage of strains agglutinated from each source would have been the same. The above findings might therefore be taken to indicate dissimilarity of origin. All that can be said on this point is that a very large number of dissimilar vibrio types exists and that therefore a correspondingly large number of strains would have to be examined to ensure that the results were not due to selection of material. In this series even amongst the Bengal strains it is unfortunate that, while nearly all the case strains were obtained from the inhabitants of Calcutta itself, the carrier and water strains were obtained chiefly from Diamond Harbour some miles outside Calcutta. It is possible also that certain vibrio types may find it easier to multiply and survive in the gut than others, though there is no evidence on this point.

On all other counts it appears that there is little to support the idea that the inagglutinable vibrios studied are causally related to any epidemic disease. The heterogeneity of species actually isolated from cases, the absence of a series of cases due to one serological type, the multiplicity of types that occur in one case, the actual identity of types in all three sources, even when no connection with cholera can be demonstrated, and the presence of these vibrios in healthy carriers without any symptoms of disease, all indicate a *chance* rather than a *causal* relationship with disease. The epidemiological features discussed in the succeeding paper support this conclusion.

With regard to any possible relationship of the inagglutinable strains in this series to the agglutinable, there is no definite evidence in this work. Two sera prepared against the classical Inaba strain, the 'H' + 'O' serum and that prepared against the derived fraction reported above, have the power of agglutinating both the classical vibrio and many of the cholera-red positive inagglutinable strains. While the cholera-red reaction is common to both agglutinable and inagglutinable strains that react with these sera, the Voges-Proskauer test shows a difference between them. A certain unpublished experiment now in progress on the survival of *V. cholerae* and the possible occurrence of changes in the vibrio during prolonged periods in conditions simulating what might occur in nature in water sources has shown that the typical agglutinable vibrio retains its characters, both serological and biochemical, unchanged for months on a pabulum of sterilized faeces and garden mould, in spite of contact with contaminating organisms. A partially rough culture containing both rough and smooth vibrios of the Inaba became eventually completely smooth.

A further point is that the strains of this series are in no case variants of the type obtainable by phage or other experimental treatment. A number of the latter strains has been dealt with by the authors. They were, with the exception of the bactericidal roughs derived by Bruce White's (*loc. cit.*) method, instable in character; they tended to revert to their parent characters; their growth characteristics were different and they have never shown any change of biochemical reaction from those of the parent strains. The strains of this series were, with one or two exceptions, completely smooth in character and as the tables show were in the large majority strains showing biochemical reactions differing from those of the true *V. cholerae*. In fact, as has been pointed out, there is no inagglutinable vibrio in the series that has all the biochemical reactions identical with those of the classical strain.

#### CONCLUSIONS.

All the evidence obtained during the course of these studies tends to indicate that the vibrios of serological type differing from the true *V. cholerae* do not produce cholera and, in addition, the characters of these vibrios and the observations made on the typical vibrios would not suggest that it is likely that the inagglutinable strains can develop into the typical agglutinable form. The extent to which these inagglutinable strains prevail in an endemic area such as Bengal and their considerable incidence in cases showing choleraic symptoms have presented a problem in India which does perhaps not exist to the same extent elsewhere and it has been necessary to clear up their significance. The opinion

of the authors is that, so far as true cholera is concerned, they do not play any part.

#### SUMMARY.

1. The results of the serological and biochemical examination of 558 inagglutinable vibrios recently isolated from cases clinically resembling cholera, healthy carriers and water in both endemic and non-endemic areas are reported.

2. The vibrios form a very heterogeneous group, both serologically and biochemically, and 31 or more different antigenic types exist among the 56 per cent of the strains which were actually grouped on the basis of their agglutination results. Within these serological groups vibrios showing different biochemical reactions were included.

The heterogeneity, both in regard to biochemical and serological characters, was more marked in the cholera-red negative strains.

3. The classification suggested by Heiberg on the basis of sugar reactions has been extended to include other types. A second method of classification on the basis of cholera-red and modified Voges-Proskauer tests is suggested. Serological findings are more closely in accordance with the latter than the former.

4. While the cholera-red positive and the cholera-red negative inagglutinable strains are, with few exceptions, positive and negative, respectively, to the modified V-P test, the classical *V. cholerae* has been shown to be different in being cholera-red positive but negative to the modified V-P test, and the few hæmolytic strains of the El Tor type of agglutinable vibrio tested have been shown to be similar to the inagglutinable vibrio in being cholera-red positive and V-P positive.

5. There is no evidence that any vibrio of serological character other than the classical was responsible for any series of cases of cholera chronologically related. An exception to this may be the strain Rangoon Rough, but the evidence in connection with this strain is incomplete.

6. The serological groups found in this series are distributed widely over India, including non-endemic areas, and have been obtained from other countries.

7. No evidence has been obtained of variation of vibrios, with the exception of partially rough strains of the typical *V. cholerae* having been found, which showed the same rough element as can be obtained from the smooth vibrios by laboratory methods.

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## APPENDIX.

### TECHNIQUE.

*Definition of a vibrio.*—Some difficulty was experienced in deciding what should be regarded as a vibrio and what should be excluded. Finally the following minimal characters were decided on:—

Gram-negative rods monotrichate in the main, but amphitrichate and lophotrichate forms admissible,

or Gram-negative curved rods, present in varying degrees.

Actively motile.

Sugar reactions—no gas formed.

Non-chromogenic (vibrios showing a deep yellow coloration on agar were not excluded).

*Testing of material for purity.*—A most important point in our technique has been the selection of a strain for examination. All strains, in which there were any doubt as to purity, were plated out as a test for colonial purity. If colonial variation was present, the variants were plated out until variation ceased. This was usually easy where translucent variants were concerned, but with opaque variants it was less easy. Where variants continued to be found, at least three successive platings were done and in many cases more. It was often found that there was no difference other than colonial form between the colonies, but in several cases two different vibrio strains were isolated. The authors cannot too strongly urge that with freshly isolated strains, unless this is carried out, purity of type cannot be ensured and even then 'double cultures' are likely to be found later in any extensive collection.

*Storage of material.*—Midget tubes containing  $\frac{1}{4}$  per cent peptone water pH 7.6 were inoculated and incubated for 4 hours at 37°C. and then sealed in the blowpipe. In this condition the strains remain in a state of suspended animation and the culture medium remains clear or nearly clear, until the tube is opened and re-incubated. In this way strains can be preserved for 12 months or more without subculture and they resist cold far more effectively than strains kept on agar slopes. This method was first suggested by Asheshov.

*Preparation of sera.*—The technique employed was largely that of Gardner and Venkatraman (*loc. cit.*).

Against Inaba, the typical 'O' agglutinable vibrio, two sera were made, (1) 'H'+'O' serum, prepared with live agar washings and (2) Inaba 'O' serum, prepared against a dried 'O' antigen, made by Mr. Bruce White and sent to this Institute by the Standards Laboratory, Oxford, for test. These formed the basis of selection of the strains, any strain agglutinating with the latter serum being excluded. In the case of other sera normal saline agar washings were boiled at 100°C. for 1 to 2 hours and diluted with normal saline to 1 mg. per c.c., and then injected into rabbits, four doses being the normal, from 0.25 mg. to 1.5 mg.

No serum was used with a titre lower than 1/500 and the average was 1/1,000 to 1/2,000. The titre of the 'H'+'O' sera was in most cases 1/2,500, though with certain of the later series a serum with a titre of 12,500 was employed.

0.2 to 0.4 per cent carbolic was used as a preservative, this being of equal value to trikresol 0.3 per cent or glycerine 50 per cent, which were also tried, from the point of view of preventing decrease in titre. An initial decrease could not be prevented: after this the serum if kept in cold storage was fairly stable though subject to a slight progressive decrease of titre in most cases. It was found that if the deposit that forms in many sera is included in the sample employed for agglutination in some cases the titre is higher than when it is omitted.

*Preparation of bacterial suspensions.*—These were prepared from formalinized agar washings (0.25 per cent formalin in normal saline) or, in the case of rough vibrios, in one-third normal saline. Dilution was performed by opacity to about 2,000 million per c.c. and filtration carried out through sterilized cotton-wool. Except for the formation of moulds this was satisfactory. Later work was however done with biniodide of mercury in a concentration of 1/1,000 in saline of such a strength that the total salt concentration was equal to normal. This was suggested by Lieut.-Colonel R. F. Bridges, R.A.M.C., of the Enteric Laboratory, Kasauli. No moulds were formed in these suspensions and they retained their homogeneity.

*Setting up and reading the test.*—This was done by Dreyer's method and with each agglutination test a control against the homologous suspension was included to make sure of the titre of the serum and to minimize errors of dilution, as all results have been recorded in percentage reaction as compared to the homologous suspension. Preliminary tests were carried out with a single tube containing serum at 1/200 and in the later work four sera were pooled together for preliminary examination and set up so that each serum was effective in the same dilution, i.e., 1/200. This procedure minimized work and was a check on the accuracy of results.

Readings were made overnight after immersion for four hours in the water-bath at 52°C. and the remainder of the time at room temperature. All readings were done by daylight.

Results were recorded as follows:—

*Titre of final dilution showing deposition.*

full ..	..	..	..	1,000.
partial	..	..	..	1,000 p.
trace	..	..	..	1,000 trace.

*Supernatant.*

clear or nearly clear	..	..	..	1,000.
turbid	..	..	..	/1,000.

*Where a turbid supernatant is present.*

deposit—heavy ..	..	..	..	/1,000 H. D.
„ medium	..	..	..	/1,000.
„ trace ..	..	..	..	/1,000 trace in all tubes.

This system eliminated any personal factor in significant readings, as all trace readings were neglected and no reactions below 25 per cent were recorded. All 25 per cent readings have been checked. It was noted that all 'H' agglutinations were of the 'stroke' type, which indicates that the supernatant is turbid, but 'O' agglutinations were of the 'full' type with a clear or nearly clear supernatant.

In certain cases it was found that the boiled antigen serum prepared as above would agglutinate to good titre only with the boiled suspension of the homologous organism. In such cases it was usually possible to prepare a good formalinized agglutinating suspension either by plating out the strain on successive days, until a translucent colony was obtained, or by inoculating the strain into its own 10 per cent antiserum peptone water. The rationale of the latter procedure is not clear, but in several instances it has enabled a good agglutinating suspension to be prepared.

*Biochemical reactions.*—Sugar reactions were done with six sugars, viz., lactose, glucose, mannite, mannose, arabinose and saccharose, in 1 per cent steamed peptone water. Readings were taken after 24 hours and five days. Cholera-red and indol tests were done on specially tested peptone. A correct brand of peptone is essential and such a brand gives an immediate reaction in a 24-hour culture on the addition of two drops of strong  $\text{H}_2\text{SO}_4$  to 2 c.c. of culture. The indol reaction will be obtained also within 2 to 3 minutes by adding Bohme's Reagent No. 1 only on the surface of the peptone water. Slight heating hastens the reaction.

*Modified Voges-Proskauer test.*—The technique of Barritt (*loc. cit.*) has been followed. The glucose phosphate medium which was prepared according to the instructions of the Ministry of Health (Report No. 71, 1934) was distributed in  $6'' \times \frac{5}{8}''$  tubes and had an initial pH of 7.5. The tubes were inoculated rather heavily with the cultures and incubated at  $37^\circ\text{C}$ . for three days. About one c.c. of the culture was then transferred to a tube and to it was added, first, 0.6 c.c. of a 5 per cent alcoholic solution of  $\alpha$ -naphthol and then 0.2 c.c. of 40 per cent KOH solution. After shaking the tubes to mix the contents, positive reactions appear as a pink colour on the surface of the fluid in about 5 to 10 minutes which then deepens and spreads to the bottom of the tube. Negatives are usually colourless but sometimes develop a very faint brownish tinge. Results were read at the end of four hours.

In parallel with this test, the V-P test was also carried out by adding 0.25 c.c. of 40 per cent KOH solution to the rest of the culture after the transfer of 1 c.c. for the  $\alpha$ -naphthol test. The results were read after four hours and again after 24 hours.

*Test for hæmolysis.*—One c.c. of a 5 per cent saline suspension of washed goat cells was added to an equal quantity of a 48-hour old culture of the organism in 1.0 per cent peptone water (pH 7.6). After shaking the tube to mix the contents, it was kept in the incubator for two hours and then transferred to the frigidaire. The results were read on the following morning.

With each batch of strains tested three controls have always been put up, i.e., a hæmolytic culture, a non-hæmolytic culture and a saline control.

*Other tests.*—All strains were tested for motility, Gram-staining, and the appearance in broth was noted. As a criterion of vibrio structure a strain is classed as a vibrio, whenever several curved forms are found on a slide. Where curved forms were not found or where there was otherwise doubt as to the species of the organisms flagellar staining was carried out by the method of Plimmer and Paine (1921). In doubtful cases the effect on gelatin was further studied.

TABLE I.  
Showing sera used in classification.

Serial number.	Serum number.	Case, Carrier, Water.	Strain number.	Place of origin.	'H' agglutination per cent.	Heiberg type	Cholera-red.	Indol.	Modified V.P.	Hemolysis.	Date of isolation.
1	G. II	Case	N32/124	Nanking	—	I	++	++	++	+	1932.
2	G. III	"	N32/123	"	—	II	++	++	++	+	1932.
3	W. G. IV	Water	N32/109	"	—	II	++	++	++	+	1932.
4	G. V	Carrier	El Tor D19	El Tor	/100	I	++	++	++	+	1934.
5	G. VI	Case	Kasauli 73	Calcutta	/100	I	++	++	++	+	1932.
6	Burma case—Rangoon Rough	"	Rangoon Rough	Rangoon	—	II	++	++	++	+	24th April, 1933.*
7	Calcutta case 1612	"	1612	Calcutta	—	II	++	++	++	+	16th January, 1933.
8	" 324/7	"	TMCH 324/7	"	/100	II	++	++	++	+	5th July, 1935
9	" 630/3T	"	630/3T	"	—	II	++	++	++	+	22nd October, 1935.
10	" 1123/2	"	" 1123/2	"	—	II	++	++	++	+	18th March, 1936.
11	" 1805/1	"	" 1805/1	"	—	I	++	++	++	+	26th May, 1936.
12	" 543/1	"	" 543/1	"	—	I	++	++	++	+	13th September, 1935.
13	" 353/3	"	" 353/3	"	—	II	++	++	++	+	7th July, 1935.
14	" 627/29/2	"	" 627/29/2	"	—	I	++	++	++	+	16th November, 1935.
15	" 627/2	"	" 627/2	"	—	I	++	++	++	+	20th October, 1935.
16	" 1158/1	"	" 1158/1	"	/100	IV	++	++	++	+	21st March, 1936.
17	" 790/6	"	" 790/6	"	—	II	++	++	++	+	16th January, 1936.
18	" 833/2/1	"	" 833/2/1	"	—	I	++	++	++	+	26th January, 1936.
19	" 367/3	"	" 367/3	"	—	II	++	++	++	+	10th July, 1935
20	Madras case 10	"	G10	Madras	—	II	++	++	N D	N D	22nd November, 1933.
21	Lahore case 9	"	L9	Lahore	—	I	++	++	N D	N D	26th July, 1936.
22	" 3	"	L3	"	—	II	++	++	N D	N D	26th July, 1936
23	" 10/5	"	L1	"	N. D.	I	++	++	N D	N D	27th July, 1936
24	Shillong case 984	"	PTC 984	"	—	IV	++	++	+	+	October 1935
25	" 3067	"	" 3067	Assam	/100	III	++	++	++	++	20th May, 1935
26	Bengal carrier 2898	"	CTV 2898	"	/100	I	++	++	++	++	17th December, 1934.
27	" 2943	Carrier	" 2943	Bengal	/100	II	++	++	++	++	1st November, 1935.
28	" 8314	"	" 8314	"	/100	II	++	++	++	++	6th November, 1935.
29	" 5780	"	" 5780	"	—	III	++	++	++	++	9th December, 1935
30	" 5780	"	" 5780	"	—	III	++	++	++	++	2nd November, 1935
31	Bengal water 4B	Water	W. Tank 4B (1785)	"	—	II	++	++	++	++	1st November, 1935
32	" 1A	"	W. Tank 1A (2074) T	"	/100	II	++	++	++	++	8th January, 1936.
33	Kohat original ..	"	W. K. O.	Kohat	N. D.	V	++	++	++	++	February 1934

\* This is a strain isolated from a case of cholera, originally showing rough colonial characters but now typically smooth  
 N. D. = Not done      — = Negative      + = Positive.

TABLE II.

*Distribution of serological types by periods and source.*

BENGAL EPIDEMIC YEAR, 1935-36.																		
Serial number.	Strain number.	CASL.				CARRIER.				WATER.				TOTAL.				
		Post-epidemic period.	Pre-epidemic period.	Epidemic period.	Total.	Post-epidemic period.	Pre-epidemic period.	Epidemic period.	Total.	Post-epidemic period.	Pre-epidemic period.	Epidemic period.	Total.	Post-epidemic period.	Pre-epidemic period.	Epidemic period.	Total.	
1	Burna case—Rangoon Rough.	..	..	20	20	..	3	..	3	..	..	..	..	..	3	20	23	
2	G. III ..	..	1	5	6	1	..	3	4	2	1	3	1	3	3	9	13	
3	Calcutta carrier 2898	} 1	2	9	12	..	2	1	3	..	3	..	3	1	7	10	18	
4	" case 324/7		..	..	..	1	4	3	8	..	..	2	5	1	7	5	13	
5	" carrier 2943		..	..	2	2	..	..	..	..	..	..	..	..	..	2	2	
6	" case 1612	..	..	6	6	..	..	..	..	..	..	..	..	..	..	6	6	
7	G. II ..	..	..	2	2	..	4	2	6	..	3	..	3	..	7	4	11	
8	Bengal water 4B	..	..	..	..	..	1	..	1	..	..	..	..	..	1	..	1	
9	Madras case 10	..	..	..	..	2	4	..	6	..	1	2	2	2	5	1	8	
10	Calcutta case 630/3T	..	..	1	1	..	1	2	3	..	..	1	1	..	1	4	5	
11	Lahore case 9	..	..	..	..	1	6	..	7	2	1	3	1	1	8	1	10	
12	Calcutta carrier 8314	..	..	..	..	..	1	1	2	..	2	1	3	..	3	5	8	
13	Bengal water 1A	..	..	3	3	..	..	..	2	..	..	1	3	..	..	7	8	
13	Calcutta case 1123/2	..	..	6	6	1	..	..	1	..	..	1	1	1	..	7	8	

[illegible]

TABLE II—*concl'd.*

Serial number.		Strain number.		BENGAL.					NOT BENGAL.					ALL SOURCES.					
				OUTSIDE EPIDEMIC YEAR, 1935-36.		TOTAL ALL PERIODS.			TOTAL ALL PERIODS.			TOTAL ALL PERIODS.							
				Case.	Carrier.	Water.	TOTAL.	Case.	Carrier.	Water.	Others.	TOTAL.	Case.	Carrier.	Water.	Others.	TOTAL.		
1	Burma case—Rangoon Rough.	4	..	..	4	24	3	..	..	2	..	..	1	3	26	3	..	1	30
2	G. III ..	4	5	1	10	10	9	4	..	3	10	4	..	17	13	19	8	..	40
3	Calcutta carrier 2898	5	..	..	5	17	3	3	..	3	6	8	..	17	20	9	11	..	40
	" case ! 324/7		..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
4	" carrier 2943	1	..	..	1	1	8	5	..	1	1	1	..	3	2	9	6	..	17
5	" case 1612	5	..	..	5	7	..	..	..	3	6	1	..	10	10	6	1	..	17
6	G. II ..	2	..	..	2	8	..	..	..	1	1	6	..	8	9	1	6	..	16
7	Bengal water 4B	..	1	..	1	2	7	3	..	..	..	..	..	..	2	7	3	..	12
8	Madras case 10	1	..	..	1	1	1	..	..	2	6	..	..	8	3	7	..	..	10
9	Calcutta case 630/3T	2	..	..	2	2	6	2	..	..	..	..	..	..	2	6	2	..	10
10	Lahore case 9	1	1	..	2	2	4	1	1	1	1	1	1	4	3	5	2	1	11
11	Calcutta carrier 8314	..	..	..	..	..	7	3	..	10	..	..	..	1	..	7	4	..	11
12	Bengal water 1A	1	1	..	2	4	3	3	..	10	..	..	1	1	4	..	4	..	11
13	Calcutta case 1123/2	1	..	..	1	7	1	1	..	9	..	..	1	1	7	1	2	..	10
14	" " 1805/1	3	..	..	3	7	1	..	..	8	..	..	..	8	7	1	8	..	16



[illegible]

TABLE IIa.

*Incidence of case, carrier and water strains classified by sera in the three groups.*

	Case strain.	Percentage incidence.	Carrier strain.	Percentage incidence.	Water strain.	Percentage incidence.	Other strain.	Percentage incidence.	TOTAL.
Case sera (22)	113	55.4	53	26.0	36	17.6	2	1.0	204
Carrier sera (4)	2	6.7	16	55.3	12	40	..	..	30
Water sera (3)	6	19.3	13	42.0	12	38.7	..	..	31
Combined sera (2) (Case + carrier) (Case + water)	24	52.2	9	19.5	13	28.3	..	..	46
TOTALS CLASSIFIED	145	46.7	91	29.2	73	23.5	2	0.6	311
TOTALS EXAMINED	202	..	182	..	172	..	2	..	558



*Percentage incidence of serological types.*

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[illegible]

13	Calcutta case 1123/2	..	..	6-6	5-4	7-7	..	..	1-1	..	..	7-1	1-4	5-9	..	5-3	3-0
14	" " 1805/1	..	..	4-4	3-6	..	..	..	1-1	..	..	..	..	..	..	3-8	1-8
15	" " 543/1	..	25	11-1	2-7	..	..	..	..	..	..	..	..	5-9	1-6	..	1-1
16	Water vibrio Kohat original.	..	..	..	..	..	2-1	..	2-2	..	..	3-5	4-2	..	2-4	1-5	1-8
17	Lahore case 1	..	..	5-5	1-1	..	..	..	..	..	..	..	..	..	0-8	0-8	0-7
18	Calcutta case 353/3	..	..	..	1-1	..	..	..	..	..	..	..	..	..	..	0-8	0-4
19	Shillong case 984	..	..	5-5	0-9	..	..	..	..	..	1-8	..	1-4	..	1-6	..	0-7
	W. G. IV	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
20	G. VI	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
21	Calcutta case 627/29/2	..	..	5-5	0-9	..	..	..	..	..	..	..	..	..	..	..	0-4
22	" " 627/2	..	25	11-1	2-7	..	..	..	..	..	..	..	..	5-9	0-8	..	1-1
23	" " 1158/1	..	..	..	2-7	..	..	..	..	..	..	..	..	..	1-6	2-2	1-1
24	Shillong case 3067	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
25	Lahore case 10/5	..	..	..	1-1	..	..	..	..	..	..	..	..	..	..	0-8	0-4
26	" " 3	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
27	Calcutta case 790/6	..	..	5-5	0-9	..	..	..	..	..	..	..	..	..	0-8	..	0-4
28	" " 833/2/1	..	..	5-5	0-9	..	..	..	..	..	..	..	..	..	0-8	..	0-4
29	G. V	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
30	Calcutta case 307/3	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
31	" " carrier 5780	..	..	..	..	..	2-1	..	1-1	..	..	..	..	..	0-8	..	0-4
Unlabelled		..	25	33-7	29-0	29-3	53-8	41-0	46-2	..	66-5	35-9	60-7	46-9	52-2	34-1	42-8
Total		..	4	18	90	112	13	48	89	..	57	14	71	17	123	132	272

## Frequency of sampling

[illegible]

[illegible]

TABLE IV—concl'd.

Serial number.	Heiberg type of homologous strain.	WATER.										OTHERS.		TOTAL.									
		HEIBERG TYPE.												HEIBERG TYPE.									
		I.	II.	III.	IV.	V.	VI-1.	VI-2.	VII.	I aberrant.	V aberrant.	Total.	I.	II.	III.	IV.	V.	VI-1.	VI-2.	VII.	I aberrant.	V aberrant.	Total.
1	II	Burma case—Rangoon Rough.	..	..	..	..	..	..	..	..	1	1	..	30	..	..	..	..	..	..	..	..	30
2	II	G. III ..	2	3	1	..	..	..	..	..	..	..	4	33	..	1	..	..	..	..	1	..	40
3	II	Calcutta carrier 2898	2	7	..	..	2	..	..	11	..	..	3	35	..	..	2	..	..	..	..	..	40
		" case 324/7		..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
4	II	" carrier 2943	..	6	..	..	..	..	..	6	..	..	..	17	..	..	..	..	..	..	..	..	17
5	II	" case 1612	1	..	..	..	..	..	..	1	..	..	1	16	..	..	..	..	..	..	..	..	17
6	I	G. II ..	..	5	..	..	..	..	..	6	..	..	4	11	..	..	..	..	..	..	1	..	16
7	II	Bengal water 4B	..	3	..	..	..	..	..	3	..	..	..	12	..	..	..	..	..	..	..	..	12
8	II	Madras case 10	..	..	..	..	..	..	..	..	..	..	..	10	..	..	..	..	..	..	..	..	10
9	II	Calcutta case 630/3T	1	1	..	..	..	..	..	2	..	..	1	9	..	..	..	..	..	..	..	..	10
10	I	Lahore case 9	1	..	1	..	..	..	..	2	..	1	3	5	2	1	..	..	..	..	..	..	11
11	II	Calcutta carrier 8314	..	2	..	..	..	..	..	4	..	..	4	7	..	..	..	..	..	..	..	..	11
12	II	Bengal water 1A	2	2	..	..	..	..	..	4	..	..	8	3	..	..	..	..	..	..	..	..	11
13	II	Calcutta case 1123/2	..	1	1	..	..	..	..	2	..	..	1	8	1	..	..	..	..	..	..	..	10
14	I	" 1805/1	3	4	..	..	1	..	..	8	..	..	9	6	..	..	1	..	..	..	..	..	16
15	I	" 543/1	..	1	..	..	..	..	..	2	..	..	3	1	..	..	..	..	..	..	1	..	5
16	V	Water vibrio Kohat original	..	4	..	..	1	..	1	5	..	..	..	6	1	..	..	..	..	..	..	..	8



[illegible]

TABLE V.

*Showing titre and source of classified strains showing Heiberg reactions differing from normal in the serological groups.*  
 Heiberg's classification.

Serum.	CASE.					CARRIER.		WATER.			OTHERS.	
	Homologous Heiberg.	Normal Heiberg.	Atypical Heiberg.	Percentage titre.	Source.	Atypical Heiberg.	Percentage titre.	Atypical Heiberg.	Percentage titre.	Source.	Atypical Heiberg.	Percentage titre.
G. III ..	II	II	I	50	Bengal	I	50	I	100	Bengal	..	..
..	..	..	..	..	..	..	..	I	50	"	..	..
..	..	..	..	..	..	..	..	IV	100	Rajputana	..	..
..	..	..	..	..	..	..	..	I	200	"	..	..
..	..	..	..	..	..	..	..	V	100	"	..	..
Calcutta carrier 2898	..	..	..	..	..	..	..	I	25	Bengal	..	..
" case 324/7	..	..	..	..	..	..	..	I	100	Rajputana	..	..
..	..	..	..	..	..	..	..	VI <sub>1</sub>	/25	"	..	..
..	..	..	..	..	..	..	..	VI <sub>1</sub>	25	"	..	..

Calcutta case 1612	..	II	II	..	..	..	..	..	..	I	100	"	..	..
G. II	..	I	II	I	100	Bengal	..	..	..	I aberrant	25	"	..	..
"	..	..	..	..	100	"	..	..	..	..	..	..	..	..
"	..	..	..	..	100	"	..	..	..	..	..	..	..	..
"	..	..	..	..	100	"	..	..	..	..	..	..	..	..
Calcutta case 630/3T	..	II	II	..	..	..	..	..	..	I	50	Bengal	..	..
Lahore case 9	..	I	II	I	100	Bengal	..	..	..	I	50	"	III	50
"	..	..	..	..	100	"	..	..	..	IV	200	Rajputana	..	..
"	..	..	..	..	100	Lahore	..	..	..	..	..	..	..	..
Calcutta carrier 8314	..	II	II	..	..	..	I	100	Bengal	I	50	Bengal	..	..
"	..	..	..	..	..	..	I	I	50	I	50	Rajputana	..	..
Bengal water 1A	..	II	I	II	50	Bengal	..	..	..	II	25	"	..	..
Calcutta case 1123/2	..	II	II	..	..	..	I	25	Bengal	III	100	"	..	..
"	..	..	..	..	..	..	..	..	..	..	..	..	..	..

Mouse passage.



Shillong case 984	..	III	III	I aberrant	100	Shillong	..	..	..	..	IV	200	Bengal	..	..
W. G. IV	..	II	II				..	..	..	..	II	100	Nanking	..	..
G. VI	..	I	II	I	100	Nanking*	..	..	..	..	..	..	..	..	..
Calcutta case 627/29/2	..	I	I	II	100	Bengal	..	..	..	..	..	..	..	..	..
" " 627/2	..	V	II	V	100	Bengal*	..	..	..	..	..	..	..	..	..
Lahore case 10/5	..	IV	IV	II	50	"	..	..	..	..	..	..	..	..	..
" " 3	..	II	II	III	50	"	..	..	..	..	I aberrant	100	Rajputana	..	..
Calcutta case 833/2/1	..	I	I	II	25	Bengal	..	..	..	..	VI-2 VI-2	50 25	Rajputana "	.. ..	.. ..

\* Strains from which serum prepared.

† Auto-agglutinable in 0.85 per cent saline.





TABLE VII.  
*Results of fermentation tests of inagglutinable vibrios.*

Results of fermentation tests of <i>inoculum</i>																M = Mannose.						
Heiberg type :—	I.		II.		III.		IV.		V.		VI-1.		VI-2.		VII.		I aberrant.		V aberrant.		TOTAL.	
	M + S + A —	M — S + A —	M + S + A +	M — S + A +	M + S — A —	None of the sugars fermented.	0	1	2	0	0	0	0	0	0	0	0	0	0	0	0	
Case ..	36	118	30	7	7	0	1									1	2	0	0	202		
Carrier ..	37	123	16	3	1	0	2									0	0	0	0	182		
Water ..	26*	100	9	9*	4	10	2									0	9	3	3	172		
Others ..	..	1	1	..	..	..	..									..	..	..	..	2		
TOTAL ..	99	342	56	19	12	10	5									1	11	3	3	558		

\* = One strain fermented lactose.  
S = Saccharose.  
+ = Acid produced : no gas. .

M = Mannose.  
A = Arabinose.  
— = Acid not produced.



TABLE VIII.  
*Results of cholera-red test in relation to fermentation type.*

Heilberg type:—	I.	II.	III.	IV.	V.	VI-1.	VI-2.	VII.	I aberrant.	V aberrant.	TOTAL.
Cholera-red + ..	89	341	0	0	0	0	5	0	9	3	447
Cholera-red — ..	10	1	56	19	12	10	0	1	2	0	111
TOTAL ..	99	342	56	19	12	10	5	1	11	3	558

TABLE IX.

*Grouping of inaggluinable vibrios according to the results of cholera-red and modified Voges-Proskauer reactions.*

Heiberg type:—	C-R — V.P —								EXCEPTIONS.					TOTAL.				
	C-R + V.P +				C-R — V.P —				C-R — V.P +									
	I.	II.	VI <sub>2</sub> .	III.	IV.	V.	VI <sub>1</sub> .	VII.	I.	II.	I aberrant.	II.	VI <sub>2</sub> .		I aberrant.	V aberrant.	I.	III.
Case ..	22	59	1	28	7	7	0	1	1	1	2	1	0	0	0	4	1	135
Carrier ..	29	65	2	15	2	1	0	0	1	0	0	1	0	0	0	1	0	117
Water ..	11	51	0	5	7	1	8	0	1	0	0	1	2	7	3	2	0	99
TOTAL ..	62	175	3	48	16	9	8	1	3	1	2	3	2	7	3	7	1	351
328																		23

C-R = cholera-red. V-P = modified Voges-Proskauer reaction.

TABLE X.  
*Results of the gelatin liquefaction test in relation to fermentation types.*

Heiberg type:—	I.	II.	III.	IV.	V.	VI-1.	VI-2.	VII.	I aberrant.	V aberrant.	TOTAL.
Liquefied ..	35	34	52	14	11	2	5	1	11	3	168
Not liquefied ..	..	..	..	5	..	8	..	..	..	..	13
TOTAL ..	35	34	52	19	11	10	5	1	11	3	181

TABLE XI.

*Serological groups according to geographical source.*

Serum.	BENGAL.				LAHORE.	MADRAS.			MANDAPAM.	RAJPUTANA.	SHILLONG.	NANKING.		
	Case.	Carrier.	Water.	Total.	Case.	Case.	Water.	Total.	Carrier.	Water.	Case.	Case.	Water.	Total.
1 Burma case—Rangoon Rough.	24	3	..	27	1	..	..	..	..	..	..	..	..	..
2 G. III ..	10	9	4	23	2	..	..	..	10	4	1	..	..	1
3 Calcutta carrier 2898	17	3	3	23	1	1	3	4	6	5	..	..	..	..
"   case 324/7 ..		8	5	14	1	..	..	..	1	1	..	..	..	..
4 Calcutta carrier 2943 ..	1	8	5	14	..	3	..	3	6	1	..	..	..	..
5 "   case 1612 ..	7	..	..	7	..	..	..	..	..	1	..	..	..	1
6 G. II ..	8	..	..	8	..	..	5	5	1	1	1	..	..	..
7 Bengal water 4B ..	2	7	3	12	..	..	..	..	..	..	..	..	..	..
8 Madras case 10 ..	1	1	..	2	..	2	..	2	6	..	..	..	..	..
9 Calcutta case 630/3T ..	2	6	2	10	..	..	..	..	..	..	..	..	..	..
10 Lahore case 9 ..	2	4	1	7	1	..	..	..	1	1	..	..	..	..
11 Calcutta carrier 8314 ..	..	7	3	10	..	..	..	..	..	1	..	..	..	..
12 Bengal water 1A (2074T)	4	3	3	10	..	..	..	..	..	1	..	..	..	..
13 Calcutta case 1123/2 ..	7	1	1	9	..	..	..	..	..	1	..	..	..	..
14 "   "   1805/1 ..	7	1	..	8	..	..	2	2	..	6	..	..	..	..
15 "   "   513/1 ..	..	..	..	3	..	..	..	..	..	2	..	..	..	..

[illegible]

TABLE XI—*concl.*

Serum.!	KARACHI.	POONA.	BASRAH.	RANGOON.	EL TOR.	BATAVIA.	KOHAT.	RAWAL-PINDI.	BANNU.	BANGKOK.	Others.	TOTAL.
	Case.	Case.	Case.	Case.	Carrier.	Case.	Water.	Water.	Dysentery case.	Water.		
1 Burma case—Rangoon Rough.	..	..	..	1	..	..	..	..	..	..	1	30
2 G. III ..	..	..	..	..	..	..	..	..	..	..	..	40
3 Calcutta carrier 2898	1	..	..	..	..	..	..	..	..	..	..	40
4 " case 324/7	..	..	..	..	..	..	..	..	..	..	..	17
5 " carrier 2943	..	..	..	..	..	..	..	..	..	..	..	17
6 " case 1612	..	..	..	..	..	..	..	..	..	..	..	16
7 G. II ..	..	..	..	..	..	..	..	..	..	..	..	12
8 Bengal water 4B	..	..	..	..	..	..	..	..	..	..	..	10
9 Madras case 10	..	..	..	..	..	..	..	..	..	..	..	10
10 Calcutta case 630/3T	..	..	..	..	..	..	..	..	..	..	..	11
11 Lahore case 9	..	..	..	..	..	..	..	..	..	..	1	11
12 Calcutta carrier 8314	..	..	..	..	..	..	..	..	..	..	..	11
13 Bengal water 1A (2074T)	..	..	..	..	..	..	..	..	..	..	..	10
14 Calcutta case 1123/2	..	..	..	..	..	..	..	..	..	..	..	16
15 " " 1805/1	..	..	..	..	..	..	..	..	..	..	..	5
16 " " 543/1	..	..	..	..	..	..	..	..	..	..	..	8
Water vibrio Kohat original	..	..	..	..	..	..	1	..	..	..	..	

[illegible]





## A NOTE ON THE BACTERIOLOGICAL FINDINGS IN CLINICAL CHOLERA IN CALCUTTA IN RELATION TO EPIDEMIOLOGY.

BY

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THE systematic bacteriological examination of cases, diagnosed clinically as cholera in Calcutta, has shown results which differ widely from those obtained in the examination of cases during epidemics elsewhere. It is the usual experience that when positive isolations are obtained from cholera cases during an epidemic the major proportion of the vibrios are typical in their serological and other characters. In Calcutta where cholera is endemic it has been found that from a considerable number of cases with choleraic symptoms only inagglutinable vibrios are isolated. It is of considerable importance to determine the significance and the ætiological rôle of these vibrios. This subject has been considered from the bacteriological point of view in the preceding communication (Taylor, Pandit and Read, 1937) and it is now proposed to discuss the subject from the epidemiological point of view.

The material on which this paper is based consists of the findings at the Campbell Hospital, Calcutta, during the years 1934-36 and also on the cholera figures supplied by the Health Officer, Calcutta Municipality. The characters of the vibrios obtained from 107 of the 139 cases of the epidemic year 1935-36, from which inagglutinable vibrios only were isolated, have been given in the previous communication (Taylor, Pandit and Read, *loc. cit.*).

The findings from these sources are shown in the Table. It will be seen that cholera cases are recorded continuously throughout the period and for the purpose of convenience their incidence has been classified in 4-weekly periods. Three seasonal periods, post-epidemic, pre-epidemic and epidemic, are demonstrated on the following basis. While cases occur throughout the year there is a definite period in each year in which a slight increase in the number of cases first occurs and this rise is followed later by a marked increase. The latter sharp rise is found

in the case of the Campbell Hospital admissions to occur before cases reach the limit of 100 per 4-weekly period in both of the years for which figures are available. This figure has been taken as demarcating the commencement and termination of the epidemic period in both years. This is of course an arbitrary level and would not necessarily be correct for other years. The remainder of the year was divided up on the basis of the observation that at a certain period the number of agglutinable vibrio cases occurring in the Campbell Hospital suddenly increased. In the year 1934 the rise was from 11 per cent of the total cases admitted to 46 per cent in one 4-weekly period, in 1935 from 6 to 42 per cent. In 1934 this occurred 8 weeks before the onset of the epidemic period and in 1935, 16 weeks. This enabled the inter-epidemic period to be divided into two halves, in which, as far as the figures are concerned, it appears that the bacteriological condition in the stools of the patient is different.

That the cases were in actual fact of a different nature during the two halves of the inter-epidemic period is also suggested by the mortality rate of all cases which rose from 7 per cent in the post-epidemic period of 1934 to 19 per cent in the pre-epidemic period and from 6 per cent to 20 per cent in 1935.

If the Health Officer's figures are studied it will be seen that the findings are very much the same. Perhaps the figures that provide the least discrepancy are those of general incidence. Here a greater proportional number of cases is admitted to hospital when the total number of cases is low. This is due to the increased difficulty of dealing with cholera cases at epidemic periods. It is important to note, however, that there is the same gradual rise that occurs at the different 4-weekly periods, a slow rise being followed after 8 to 16 weeks by a rapid rise that heralds the onset of the epidemic period.

With regard to the incidence of deaths the lowest divergence between the two sets of figures is during the pre-epidemic period, although the number of cases per period is higher than in the post-epidemic period when the divergence is greater. This is due to the low mortality of cases in the Campbell Hospital figures during the post-epidemic periods.

There is a considerable discrepancy between the mortality rate of the two sets of figures but the seasonal variation of the two sets is of the same order. In parallel with the figures for death incidence the least discrepancy is found in the pre-epidemic period and the greatest in the post-epidemic period. It appears that during the pre-epidemic period the effects of hospital treatment are less marked than at other times, especially so than during the post-epidemic periods. In other words a severer type of case is being admitted. This is not a question of the hospital staff being less able to give attention to these cases as the total number of cases is not markedly increased in the pre-epidemic period and is certainly not so great as during the epidemic period when the discrepancy is only a little greater. It seems that there is a difference in the *type of case* occurring in the post-epidemic period especially amongst those admitted to the Campbell Hospital, as the difference in the Health Officer's figures is less striking.

It is concluded that allowing for the above factors no significant divergence between the admission rate of the Campbell Hospital and the figures for cholera in the general population can be demonstrated.

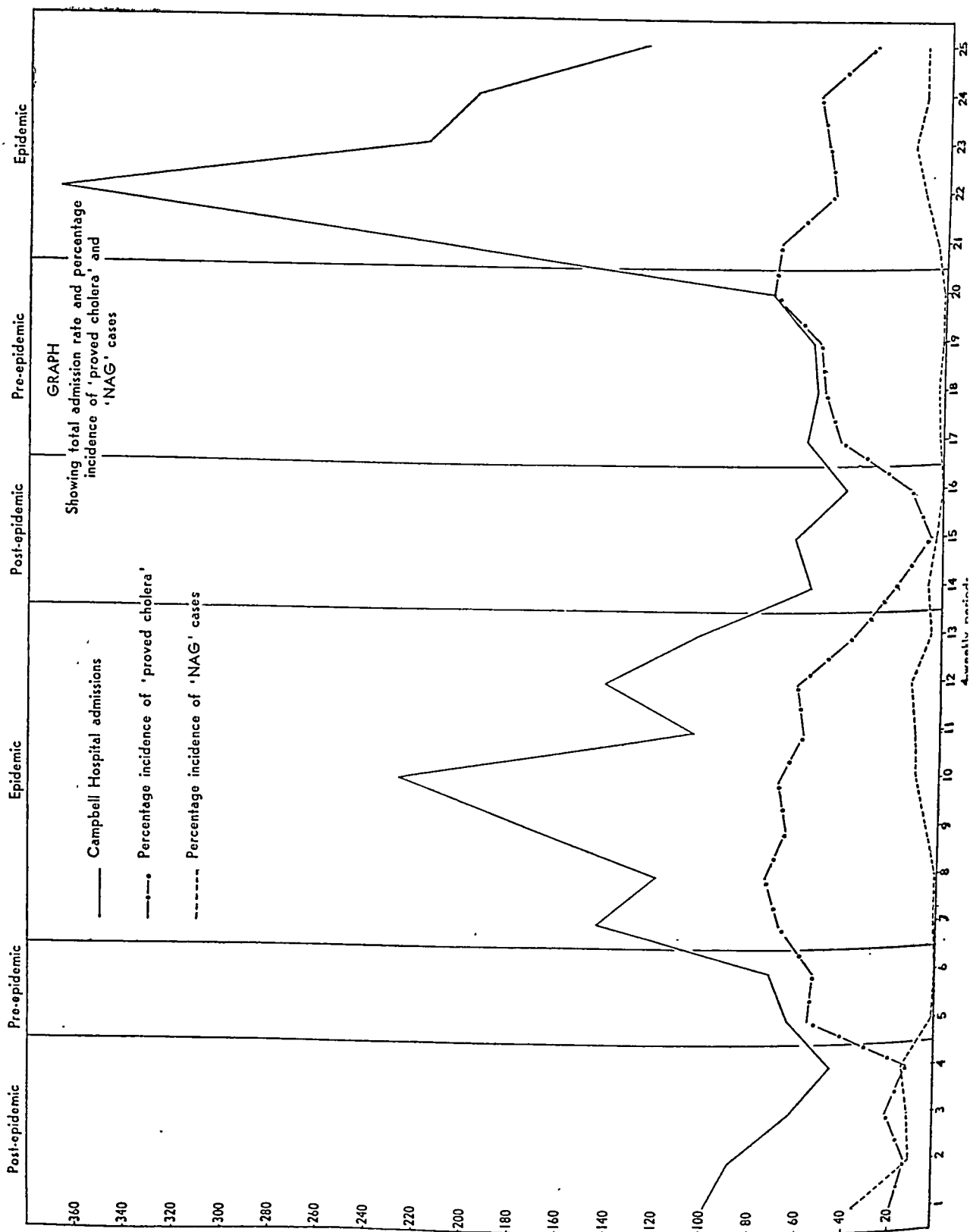
Turning to the Campbell Hospital figures it will be seen that these are divided up into :—

- (1) Agglutinable vibrio cases.
- (2) Inagglutinable vibrio cases, in which inagglutinable vibrios only are isolated.
- (3) Bacteriologically negative cases.
- (4) Cases unexamined.

A further division is added by the author by combining the agglutinable vibrio cases with those of the ' fatal cases unexamined '.

The validity of these groups may be considered on the following basis. It is certain that the proportion of the agglutinable vibrio cases is below the true incidence. A proportion of the other groups properly belongs to this group. The most obvious group involved is that of the ' cases unexamined '. The level of the mortality rate in this group makes it certain that the chief reason for non-examination of the stools bacteriologically was that the patients were moribund on admission. On the other hand nearly 25 per cent of cases ' not examined ' did not prove fatal. If the total number of non-fatal cases ' not examined ' is plotted for each period it will be seen that the actual numbers vary from 7 to 49. The two highest figures are 26 and 49. In both cases this rate is explained by the fact that in one 4-weekly period in each seasonal period an unusually high percentage of cases unexamined as compared with the seasonal average was non-fatal. There is in fact some considerable variation in the proportion of fatal cases from week to week and period to period. The explanation of this is probably that some of the cases were not examined for administrative reasons. Therefore nothing can be concluded as to the nature of non-fatal cases. It is not unreasonable to suppose, however, that the large majority of fatal cases unexamined would have been ' agglutinable vibrio cases ' had they been examined, not that it is always possible to make isolations in very severe cases, but the more general prevalence of the agglutinable vibrio group as compared with other forms in cases of high mortality makes it more likely that these cases would have fallen into that group. For this reason the agglutinable vibrio cases and the fatal cases not examined have been grouped together in one of the divisions of the Table.

In regard to the inagglutinable vibrio cases it is possible that a proportion of these too fall really into the agglutinable vibrio group. The known difficulties in the isolation of agglutinable vibrios in many cases, in which isolation is successful, do not justify the assumption at present that failure to isolate the agglutinable vibrio is proof of its absence. The conditions required for the successful isolation of the two vibrios do not differ ; each sample of material examined is usually small and unless many specimens are examined, it would be possible to miss the agglutinable vibrio without difficulty. In culture media moreover most inagglutinable vibrios will multiply with greater rapidity than the agglutinable and the detection of the latter is, therefore, a matter of greater difficulty when the inagglutinables are present. It is, however, by no means impossible that this group includes cases that are suffering from some infective agent, which is neither the agglutinable nor the inagglutinable vibrio, in spite of the fact that the latter is present in the stools.



The coincidence of diarrhoea and inagglutinable vibrios in the stool cannot be accepted as justification for the diagnosis of cholera. In any case whatever view is adopted as to the nature of these cases the mortality rate as compared with the agglutinable vibrio cases shows them to be cases of a very different type.

A further point with regard to the inagglutinable vibrios is their seasonal incidence. This is at a relatively high percentage figure in the first post-epidemic period and the two epidemic periods (7 to 20 per cent). In the two pre-epidemic periods and the second post-epidemic period [which is that which applies to the vibrios examined by Taylor, Pandit and Read (*loc. cit.*)] the figures are low (1 to 3 per cent). The isolation rate for the whole two years is 9 per cent. Figures of this level represent an average sampling of the general population and the incidence of these strains is of the order quoted by Couvy (1933), viz., 13.9 per cent for the vicinity of Calcutta and the figures found by Tomb and Maitra (1926) for a mining district in Bengal, viz., 30 per cent. If the report by Couvy be consulted it will be found that a similar incidence of inagglutinable vibrios in carriers has been reported elsewhere in India and in other parts of the world, where cholera was prevalent. Even if these figures be considered too high, it is to be remembered that the diarrhoeal condition of the stools of the patients will facilitate vibrio isolation.

Judging from their mortality rate the bacteriologically negative cases are in a similar position to the inagglutinable vibrio cases. Their percentage incidence differs, as being a large group they are necessarily inversely proportional to the other large group, i.e., the agglutinable vibrio cases. They too must include missed cases of agglutinable vibrio cholera and possibly it is amongst these that the fatal cases are to be found.

Considering the combined group agglutinable vibrio cases plus fatal cases unexamined, which it is considered represents a group of *proved* cholera cases, it will be seen that the increase in the percentage incidence of these cases is even more marked at the commencement of the pre-epidemic period than with the agglutinable vibrio cases alone. At the same time the mortality rates throughout the year are sufficiently uniform to justify the conclusion that even in the post-epidemic period there is no difference in the type of proved cholera case that is occurring.

The question remains as to the status of the other cases. It will be clear from the above findings alone, apart from the findings of Taylor, Pandit and Read (*loc. cit.*), that there is a likelihood that the presence of these vibrios in the stools of diarrhoeal patients is merely an expression of the condition of the stools in the general population. That they include cases of true cholera is likely, but that the inagglutinable vibrio is in any way concerned will require definite proof, if it is to be worthy of acceptance.

#### SUMMARY.

1. The epidemiological conditions under which certain of the case vibrios from the Campbell Hospital, Calcutta, dealt with in a preceding communication (Taylor, Pandit and Read, *loc. cit.*), were isolated are considered. As a control the figures for one other epidemic year and those from the Calcutta Municipality for the two years are included.

2. The figures of the Campbell Hospital and those of the Calcutta Municipality are in consonance. The greatest divergence is in the cases occurring after the true epidemic seasons.

3. The epidemic year has been divided into three seasonal periods, post-epidemic, pre-epidemic and epidemic, which are distinguished on the basis of total incidence, mortality and percentage rate of isolation of the typical agglutinable vibrios. The pre-epidemic period is defined by a change in conditions characterized by an increase in the mortality rate, together with an increase in the percentage isolation of the typical *V. cholerae* without any marked increase in the total number of cases occurring.

4. The value of this division into seasonal periods however depends on the nature of the cases from which agglutinable vibrios are not isolated. These have a low mortality rate and many may not be connected with epidemic cholera.

5. The mortality rate of cases of 'proved cholera' does not vary materially throughout the year under hospital conditions.

6. Throughout the period of observation a proportion of cases diagnosed clinically as cholera showed inagglutinable vibrios only. These have a maximum percentage incidence in the two epidemic periods and also in one post-epidemic period and are at a very low figure in both pre-epidemic periods and one post-epidemic period. Their incidence is not above the reported figures of incidence in the general population in cholera areas.

The causal relationship of these vibrios to cases of cholera is thus not demonstrated.

The whole of the cholera figures analysed in this paper were provided by Captain Pasricha, I.M.S., of the Campbell Hospital, and Dr. Biswas, Health Officer of the Calcutta Municipality, to whom acknowledgments are due for permission to publish this analysis of their figures.

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 TOMB, J. W., and MAITRA, G. C. (1926) *Ind. Med. Gaz.*, **61**, p. 537.

TABLE.

*Incidence, deaths, and mortality rates in cholera cases in the Calcutta Municipality and Campbell Hospital, Calcutta, by periods.*

Season.	Serial number.	Weekly period ending	INCIDENCE.				DEATHS.				MORTALITY.			
			H.O.'s incidence.	Campbell Hospital incidence.	Ratio.	Seasonal ratio.	H.O.'s deaths.	Campbell Hospital deaths.	Ratio.	Seasonal ratio.	H.O.'s per cent mortality.	Campbell Hospital per cent mortality.	Ratio.	Seasonal ratio.
Pre-epi- demic.	1	4-9-34	175	99	1.77	..	50	7	7.14	..	29	7	4.14	..
	2	1-10-34	140	89	1.57	..	35	7	5.00	..	25	8	3.13	..
	3	29-10-34	115	64	1.80	..	27	5	5.40	..	23	8	2.88	..
	4	26-11-34	85	47	1.81	1.72	20	1	20.00	6.6	24	2	2.92	3.77
	5	29-12-34	130	65	2.00	..	55	13	4.23	..	42	20	2.10	..
	6	21-1-35	170	73	2.33	2.17	69	13	5.31	4.77	41	18	2.28	2.16
Epidemic.	7	18-2-35	459	145	3.16	..	188	30	6.26	..	41	21	1.95	..
	8	18-3-35	673	121	5.56	..	338	23	14.70	..	50	19	2.63	..
	9	25-4-35	658	174	3.78	..	266	33	8.06	..	40	19	2.11	..
	10	13-5-35	910	227	4.01	..	421	48	8.77	..	46	21	2.19	..
	11	10-6-35	688	107	6.43	..	258	19	13.58	..	38	18	2.11	..
	12	8-7-35	790	143	5.52	..	282	17	16.59	..	36	12	3.00	..
	13	5-8-35	387	106	3.65	4.46	177	14	12.64	10.49	46	13	2.70	2.47

H. O. = Health Officer, Calcutta Municipality.

N.B.—The term 'ratio' indicates the relation of the Health Officer's figures to those of the Campbell Hospital.

TABLE—*contd.*

		INCIDENCE.				DEATHS.				MORTALITY.								
Season.	Serial number.	Weekly period ending	H.O's incidence.		Campbell Hospital incidence.		Ratio	Seasonal ratio.	H.O's deaths.	Campbell Hospital deaths.		Ratio.	Seasonal ratio.	H.O's per cent mortality.	Campbell Hospital per cent mortality.		Ratio.	Seasonal ratio.
Post-epidemic.	14	2-9-35	160	..	59	..	2.71	..	54	..	2	..	27.00	..	34	3	11.33	..
	15	30-9-35	149	..	66	..	2.26	..	19	..	5	..	3.80	..	13	8	1.36	..
	16	28-10-35	90	399	45	170	1.89	2.35	17	90	4	11	4.25	8.18	19	9	2.11	3.83
Pre-epidemic.	17	25-11-35	114	..	62	..	1.84	..	21	..	12	..	1.75	..	18	19	1.00	..
	18	2 12-35	121	..	58	..	2.09	..	55	..	14	..	3.93	..	45	24	1.88	..
	19	20-1-36	122	..	60	..	2.03	..	47	..	12	..	3.92	..	38	29	1.90	..
	20	17-2-36	206	563	77	257	2.68	2.19	89	212	13	57	6.85	3.72	43	17	2.53	1.90
Epidemic.	21	16-3-36	629	..	219	..	2.87	..	240	..	37	..	6.49	..	38	17	2.23	..
	22	13-4-36	803	..	368	..	2.18	..	256	..	37	..	6.92	..	32	10	3.20	..
	23	11-5-36	736	..	218	..	3.38	..	288	..	36	..	8.00	..	39	17	2.30	..
	24	8-6-36	850	3,018	198	1,003	4.29	3.01	359	1,143	32	142	11.22	8.05	42	16	2.63	2.71
	25	7-7-36	..	..	130	1,133	..	..	..	..	16	158	..	..	..	12	15	..

H. O. = Health Officer, Calcutta Municipality.

N.B.—The term 'ratio' indicates the relation of the Health Officer's figures to those of the Campbell Hospital.



TABLE—*contd.*

Season.	Serial number.	A.G. + FATAL CASES UNEXAMINED.				A.G. CASES.				N.A.G. CASES.					
		Deaths.		Percentage mortal-ity.	Total.	Percentage in- cidence.	Deaths.	Percentage mortal-ity.	Total.	Percentage in- cidence.	Deaths.	Percentage mortal-ity.			
		Total.	Percentage in- cidence.												
Post-epidemic.	1	4-9-34	19	19	6	32	15	15	2	13	36	36	0	0	..
	2	1-10-34	12	13	4	33	9	10	1	11	10	11	0	0	..
	3	29-10-34	14	22	3	21	14	22	3	21	8	12	2	25	..
	4	26-11-34	6	51	1	14	5	43	11	14	0	6	7	61	20
Pre-epidemic.	5	29-12-34	37	57	12	32	30	46	5	16	1	1	0	0	..
	6	21-1-35	40	77	11	23	33	63	45	4	9	0	1	0	0
Epidemic.	7	18-2-35	102	70	25	25	89	61	..	12	13	1	..	0	..
	8	18-3-35	92	76	20	22	82	68	..	10	12	1	..	0	..
	9	25-4-35	118	68	28	24	107	61	..	17	16	10	..	1	..
	10	13-5-35	161	71	40	25	146	64	..	25	17	25	..	3	..
	11	10-6-35	65	61	14	22	61	57	..	10	16	13	..	2	..
	12	8-7-35	91	64	13	14	85	59	..	7	8	20	..	1	..
	13	5-8-35	42	40	13	30	37	35	8	21	5	75	0	7	0

A.G. = Agglutinable vibrio.

N.A.G. = Inagglutinable vibrio.

TABLE—*contd.*

		A.G. + FATAL CASES UNEXAMINED.						A.G. CASES.				N.A.G. CASES.					
Season.	Weekly period ending	Total.		Percentage in.		Deaths.		Percentage mortal-ity.		Total.		Percentage in.		Deaths.		Percentage mortal-ity.	
Post-epidemic.	11	13	..	22	..	2	..	15	..	12	..	20	..	1	..	8	..
	15	4	..	6	..	1	..	25	..	3	..	4	..	0	..	0	..
	16	7	24	16	14	4	7	57	29	3	18	6	11	0	1	0	6
Pre-epidemic.	17	30	..	48	..	11	..	37	..	26	..	42	..	7	..	27	..
	18	32	..	55	..	13	..	41	..	24	..	41	..	5	..	21	..
	19	34	..	57	..	12	..	35	..	26	..	43	..	4	..	15	..
20	59	155	77	60	11	47	19	30	50	126	65	45	2	18	4	14	
Epidemic.	21	163	..	74	..	29	..	18	..	147	..	67	..	13	..	9	..
	22	192	..	52	..	34	..	18	..	172	..	47	..	14	..	8	..
	23	120	..	55	..	24	..	20	..	105	..	48	..	9	..	9	..
	24	117	..	59	..	25	..	21	..	106	..	54	..	14	..	13	..
	25	45	637	35	56	10	122	22	19	42	572	32	51	7	57	17	10

A.G. = Agglutinable vibrio.

N.A.G. = Inagglutinable vibrio.

TABLE—*contd.*

Season.	Serial number.	Weekly period ending	BACTERIOLOGICALLY NEGATIVE CASES.					CASES UNEXAMINED.				
			Total.	Percentage in-cidence.	Deaths.	Percentage mortal-ity.	Total.	Percentage in-cidence.	Deaths.	Percentage mortal-ity.		
Post-epidemic.	1	4-9-34	42	43	1	2	..	6	6	4	75	..
	2	1-10-34	65	73	3	5	..	5	6	3	60	..
	3	29-10-34	34	53	0	0	..	8	13	0	0	..
	4	26-11-34	33	70	0	0	2	2	4	1	50	38
Pre-epidemic.	5	29-12-34	25	39	1	4	..	9	14	7	78	..
	6	21-1-35	28	38	2	17	6	12	17	7	58	67
Epidemic.	7	18-2-35	36	25	5	14	..	19	13	13	68	..
	8	18-3-35	27	22	3	11	..	11	9	10	91	..
	9	25-4-35	46	26	4	9	..	11	7	11	100	..
	10	13-5-35	32	14	5	16	..	24	11	15	63	..
	11	10-6-35	29	27	3	10	..	4	4	4	100	..
	12	8-7-35	32	22	3	9	..	6	5	6	100	..
	13	5-8-35	59	55	1	2	9	5	5	5	100	80

**TABLE—concl.**

		BACTERIOLOGICALLY NEGATIVE CASES.						CASES UNEXAMINED.						
Season.	Serial number.	Weekly period ending	Total.		Percentage in.	Deaths.		Percentage mortal-ity.	Total.		Percentage in.	Deaths.		Percentage mortal-ity.
Post-epidemic.	14	2-9-35	41	..	69	..	0	..	2	..	4	..	1	50
	15	30-9-35	52	..	79	..	8	..	9	..	14	..	1	11
	16	28-10-35	21	114	47	67	4	0	21	32	47	19	4	19
Pre-epidemic.	17	25-11-35	31	..	50	..	3	..	4	..	6	..	4	100
	18	23-12-35	18	..	31	..	6	..	14	..	25	..	8	43
	19	20-1-36	16	..	27	..	0	..	17	..	29	..	8	47
	20	17-2-36	17	82	22	32	4	12	9	44	12	17	9	100
Epidemic.	21	16-3-36	44	..	20	..	11	..	17	..	18	..	16	94
	22	13-4-36	86	..	23	..	1	..	68	..	18	..	20	29
	23	11-5-36	62	..	28	..	11	..	15	..	7	..	15	100
	24	8-6-36	57	..	29	..	11	..	11	..	5	..	11	100
	25	7-7-36	70	319	54	28	7	8	3	114	2	10	3	100

## ROUGH AND SMOOTH CHOLERA VIBRIOS IN RELATION TO THEIR MODE OF DIVISION AND GROWTH.

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SINCE Arkwright (1921) described the smooth and rough types of colonies in the *coli*-typhoid and dysentery group of organisms, this colony variation has been found to be a common feature amongst the micro-organisms in general. Thus 'S' and 'R' variations in colony types have also been observed in the cholera and cholera-like vibrios and were first studied by Shousha (1923). Recent studies have further revealed that in case of *Vibrio cholerae* the 'R' types consist of opaque colonies of different degrees of roughness varying from a slightly granular to a highly rugose surface with irregular margin, resembling crumpled morocco leather, and denoted as the 'medusa-head' type by Linton, Shrivastava and Mitra (1935), while the smooth type consists of translucent colonies with smooth surface and regular margin closely corresponding to those of the *coli*-typhoid and *Salmonella* groups of organisms (Plates XXXIV to XXXVI, figs. a to i).

The rough types of cholera vibrios mentioned above have been found to differ from the smooth ones in various respects, viz., cultural characters, chemical constitution, biochemical, serological and immunological behaviour, toxicity, metabolic activities, and surface charge. To determine whether there is also a difference in the method of growth of the individual bacteria comprising these rough and smooth colonies, the growth and manner of division of single living cells selected from both 'R' and 'S' strains have been observed under the darkground illumination. The method originally employed by Topley, Barnard and Wilson (1921) and later by Nutt (1927) in the case of the *Salmonella* group of organisms has been applied with a slight modification. In each case an individual cell or one in the first stage of division was followed as it grew into a tiny colony. A soft solid medium was selected in which the daughter cells did not move apart.

## TECHNIQUE.

*Choice of medium.*—1·5 per cent agar was first tried but was found to be too opaque for darkground illumination and microphotography and at the same time too solid for the vibrio to grow well, particularly as oxygen was practically cut off by paraffin at the edge. An 8 to 10 per cent solution of gelatin such as was used by other workers does not solidify at Calcutta temperatures and even 18 to 20 per cent gelatin was found unsuitable. After much experimentation the combination of 0·5 per cent agar and 12 to 15 per cent gelatin was found satisfactory and was used in this work. The medium can undoubtedly be improved. The best results were obtained by preparing 25 to 30 per cent gelatin and 1 per cent agar in separate tubes and then mixing them in equal proportion after melting them in a water-bath just before starting the work.

The medium could not be used at 37°C. since it melted. The observations were accordingly made at room temperature which varied from 21°C. to 22°C. Microphotographs were taken at several stages of division of each type of organism. Others have been watched and sketched with the help of the camera lucida at different stages of their growth to show the actual manner in which they undergo multiplication.

*Method.*—The vibrio strain to be tested was grown in 1 per cent peptone water for 4 to 6 hours at 37°C., and then plated on ordinary agar and incubated for 16 to 18 hours at the same temperature. Small colonies of about 0·25 mm. to 1 mm. diameter containing actively dividing organisms were thus obtained. About 2 c.c. of a medium composed of 12 to 15 per cent gelatin and 0·5 per cent agar were melted in a water-bath, cooled to about 37°C., and inoculated with a portion of young colony from the agar plate. The medium was then lightly shaken while still soft and a drop of it was placed on a clean coverslip by means of a loop 3 mm. to 4 mm. in diameter. A clean, thin, glass slide was then carefully lowered over the drop and lightly pressed flat and turned upside down. A thin, bubble-free film was thus formed, just filling the space between the coverslip and the slide. The whole manipulation had to be carried out rapidly as otherwise the medium would become solidified before the slide could be placed over the drop and the preparation become uneven or too thick. The coverslip was then ringed round with melted paraffin-wax to prevent drying. The temperature of the melted paraffin should only be just above its melting point otherwise the heat of the paraffin may kill the bacteria at the margin and melt the medium. It is also to be noted that for successful darkground illumination both the coverslips and the slides must be exceptionally clean and grease-free, thin, transparent, and dry. In this experiment slides of 1 mm. thickness were used and sterility was maintained throughout as far as practicable.

(1) SMOOTH CULTURES OF *Vibrio cholerae*.

The following smooth strains were selected for study :—

- (1) INABA—a Japanese group 'Original' type strain obtained from a cholera case and belonging to Gardner's group I (Gardner and Venkatraman, 1935).

- (2) 10404—a strain recovered from a cholera case in an endemic area, Diamond Harbour (Calcutta).
- (3) 1617—a standard strain, obtained from the Central Research Institute, Kasauli.
- (4) RANGOON SMOOTH—a strain recovered from a cholera case in Rangoon.

All belong to Linton's group I (Linton, Shrivastava and Mitra, *loc. cit.*), agglutinable by the standard anti-cholera serum and possess all the characters of a typical cholera vibrio.

Preparations of smooth cultures of these organisms were made and observed as described above. An isolated motile organism, or one just in the process of division as evidenced by the appearance of a band of constriction in the middle, was selected for study and was watched under the darkground illumination usually at intervals of 2, 4, 6, 24, and 48 hours and often at shorter intervals for the first few hours. The slides were left in the frigidaire (6°C. to 10°C.) overnight. As all the observations were made at a temperature of about 21°C. in an air-conditioned room, removal of the slide from under the microscope for incubation was not necessary, and accordingly the difficulty of readjusting the slide to its former position each time after incubation was avoided. There was a definite lag period which varied between 2 to 4 hours, but once the division started it progressed fairly rapidly and in 24 hours a small cluster of cells could be observed under the microscope.

As *Vibrio cholerae* is ordinarily highly motile, it is difficult to catch in the photographic plate single and separate individuals or one just in the process of division; for, although they do not so much as move away from the actual field, there is a constant protoplasmic movement of rhythmic character which often causes them to change their shapes and sizes (Plate XXXIII, fig. 5; Plate XXXVIII, group vii, fig. 2, and group x, fig. 4). Besides, there is a sort of scintillating and often very quick circular movement round their axes and in a comparatively soft medium they even show swimming movements. But once there have been several divisions they hardly change their position and shape and can therefore be photographed more easily at this stage. The outline can be beautifully seen when properly focused and before the actual partition appears the cell slightly elongates and in the middle of its body two definite knob-like projections protrude inward from the opposite walls (Plate XXXIII, fig. 9; Plate XXXVII, group v, fig. 2). These are probably formed by the reduplication or notching of the walls and are better marked during the process of an antero-posterior wavy movement. These two projections approach each other and coalesce forming a complete wall in the middle. Then an hour-glass-like constriction appears at that spot which remains there for varying lengths of time before the two portions either separate completely and change positions or form a somewhat longer chain and then separate. In the case of a smooth strain this chain is composed usually of not more than four individual cells attached to each other by their ends. They then slip past each other and lie side by side. This slipping may occur at the first division (Plate XXXIII, figs. 2, 3 and 4) or after one or two subdivisions so that hardly is a chain of four formed when the vibrios separate at some intercellular division into pairs or

singles or into both and come to lie side by side after the slipping movement (Plate XXXIII, fig. 5; Plate XXXVII, group iii, fig. 3, and group iv, fig. 3). Long chains which are very common with the rough strains do not occur. Thus the division and multiplication go on in the above manner and in about 24 hours a small cluster of cells is formed adherent to one another and looking more or less even and smooth in outline (Plate XXXIII, fig. 6; Plate XXXVII, groups i to iv, fig. 5).

Sometimes one or two organisms at the outer border of such a mass become unusually long, equal to about three or four times the normal length of the organism (Plate XXXVII, group iv, fig. 6, and Plate XXXVIII, group x, fig. 1); but this form is more marked amongst the organisms of intermediate character such as Rangoon rough (1) (Plate XXXVIII, group vii) which is intermediate between Rangoon smooth and Rangoon rough (2) in roughness (Seal, 1935). The flagellum was also sometimes observed under the darkground illumination but the character of its movements could not be followed nor could it be brought well on to the photographic plate.

The figures in Plate XXXVII, groups i to iv, represent the sketches of the organisms at various stages of their growth, made with the help of the camera lucida. Microphotographs of a representative smooth culture (*V. inaba*) have also been taken under similar conditions and are shown in Plate XXXIII, figs. 1 to 7; these clearly demonstrate the actual process of division and multiplication of smooth vibrio strains starting from a single cell.

## (2) ROUGH CULTURES OF *Vibrio cholerae*.

The following rough strains were selected for study :—

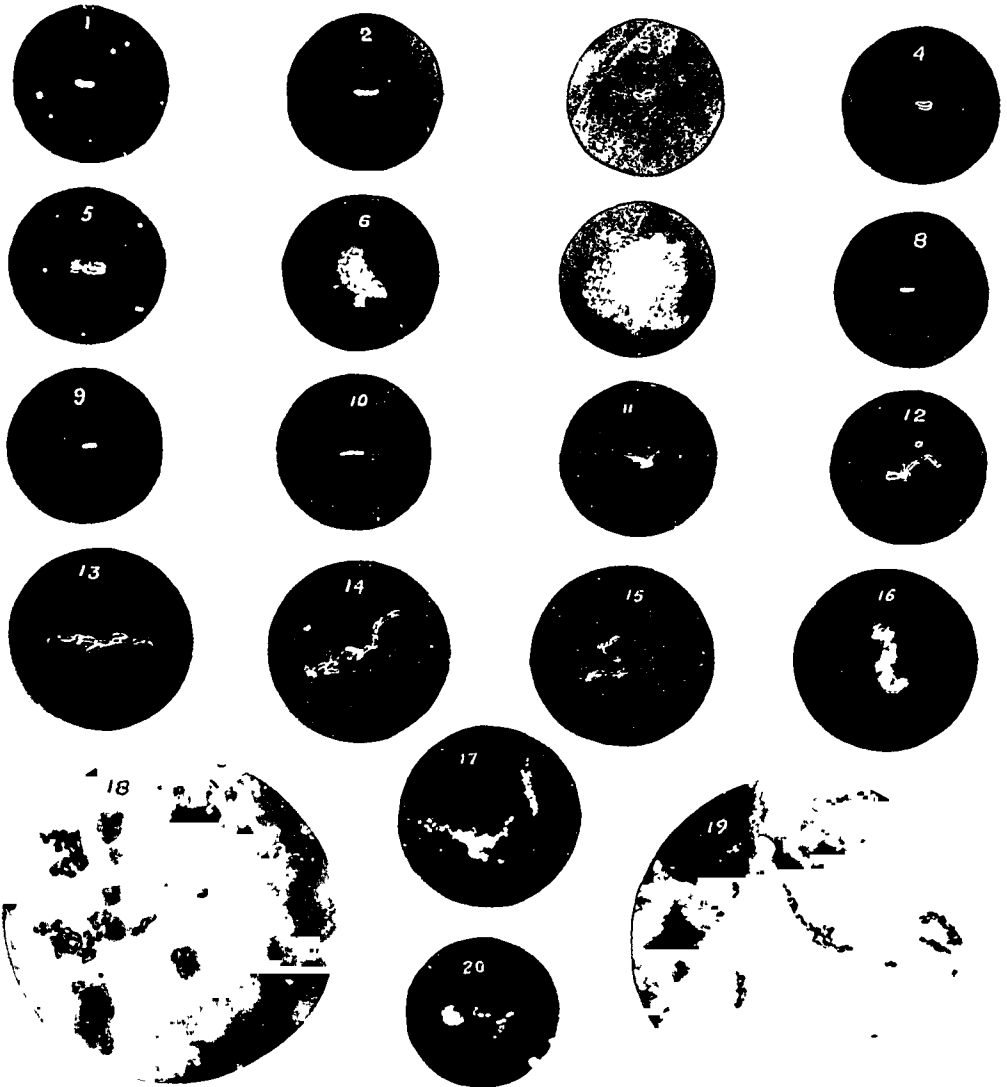
- (1) INABA 'R' (iv)—A rough dissociant of *V. inaba* obtained through the courtesy of Mr. Bruce White.
- (2) 1617 SINGLE CELL 'R'—A rough dissociant derived from 1617 single-cell subculture, kindly sent by Dr. C. G. Pandit, King Institute, Guindy, Madras.
- (3) SINGLE CELL X(1) 'R'—A rough dissociant derived from *V. X*(1) single-cell subculture, received from Lieut.-Colonel Anderson, I.M.S., Pasteur Institute, Shillong (Assam).
- (4) RANGOON 'R' (1)\*—A rough vibrio isolated from a plate which had been streaked with stool from a cholera case in Rangoon.
- (5) RANGOON 'R' (2)—A 'medusa-head' dissociant derived from Rangoon 'R' (1) subculture by Linton *et al.* (*loc. cit.*).

All the above strains are typically rough, non-agglutinable by the standard anti-cholera serum and show variable characters unlike the typical cholera vibrios.

\* The strain which has been designated 'Rangoon rough' has in time developed different characters in the hands of different workers. In our hands it has the rough characters indicated, while in other laboratories it is reported now to be a typically smooth strain of different serological type from the classical *V. cholerae*.



PLATE XXXIII.  
(Microphotographs.)



Figs. 1 to 7—showing the various stages of growth of a single *smooth* vibrio cell taken at intervals of 2 to 48 hours.

Figs. 8 to 19—showing the various stages of growth of single *rough* vibrio cells taken at intervals of 2 to 48 hours.

Fig. 20—showing *rough* vibrio cells with opaque and thick ends.

PLATE XXXIV.

(Microphotographs.)

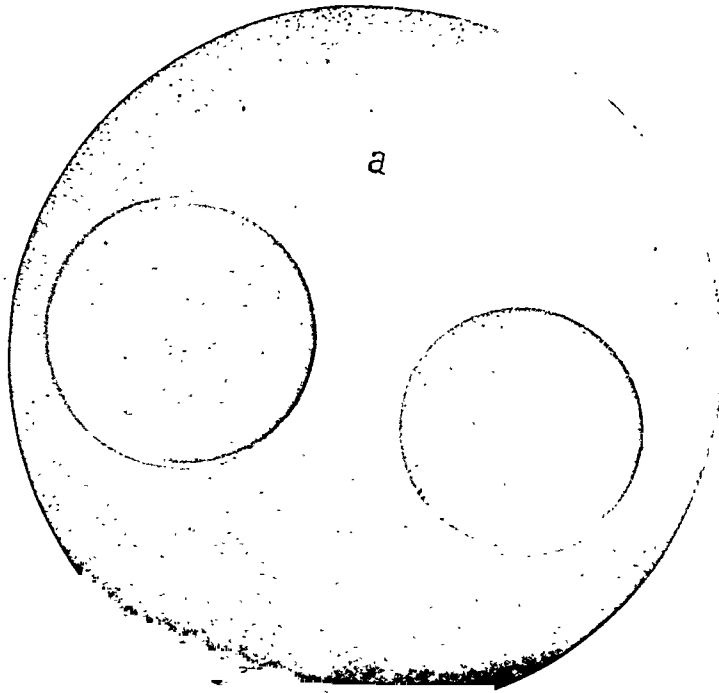


Fig. a.—Typical *smooth* vibrio colonies.



Fig. b.—Early *rough* stage of vibrio with smooth colonies and showing finely granular surface.

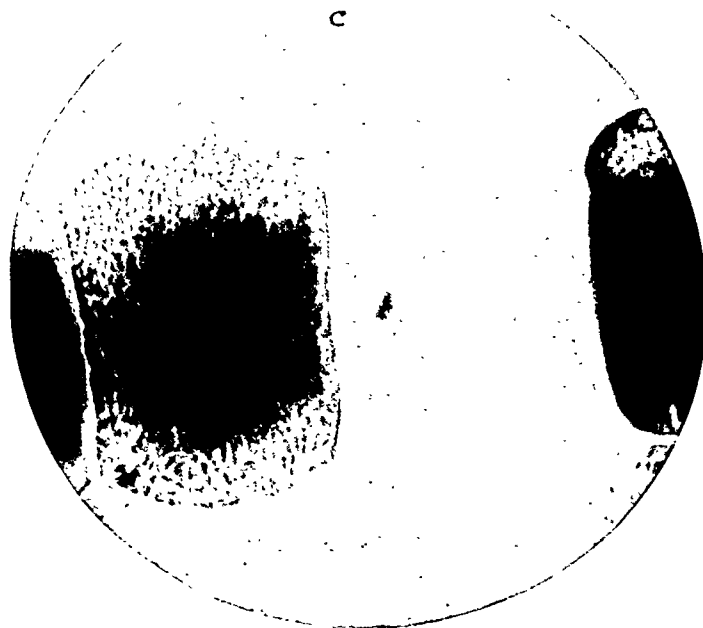


Fig. c.—*Rough* vibrio colonies mixed with smooth colonies and showing coarsely granular surface.

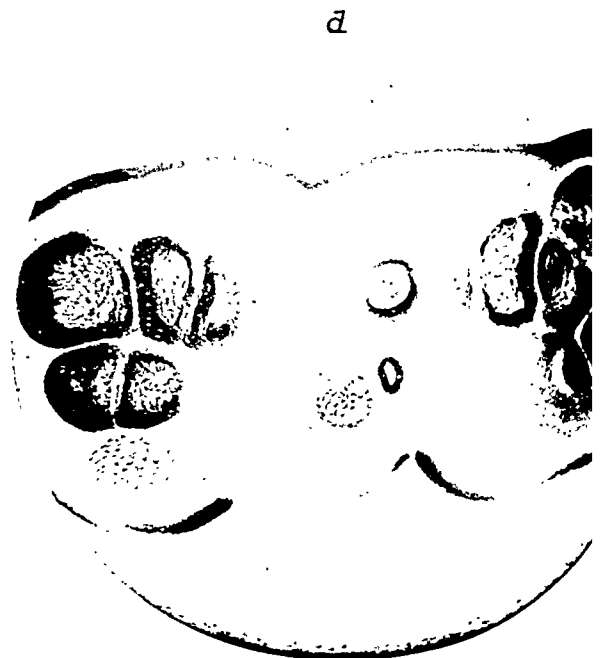


Fig. d.—*Rough* vibrio colonies superimposed on smooth colonies and showing rugose surface.

PLATE XXXV  
(Microphotographs.)

e



—A conjoined vibrio colony of which only the central rough portion appeared in 24 hours and was surrounded by the smooth portion in 48 hours.

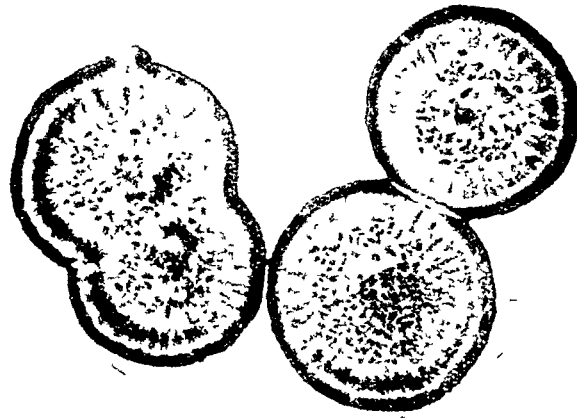


Fig. f.—Rough vibrio colonies characterized by radiating folds and 'seed-like' deposits in the centre. A small smooth dissociant is seen peeping out from one of them.

g



g.—A mixture of smooth and 'medusa-head' rough colonies.

h

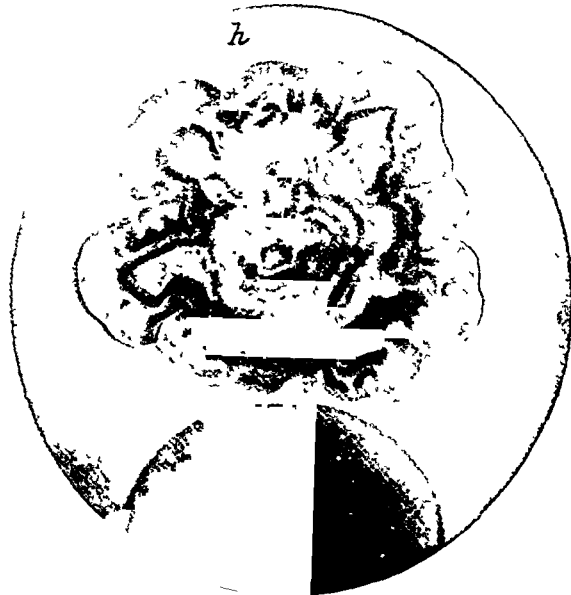
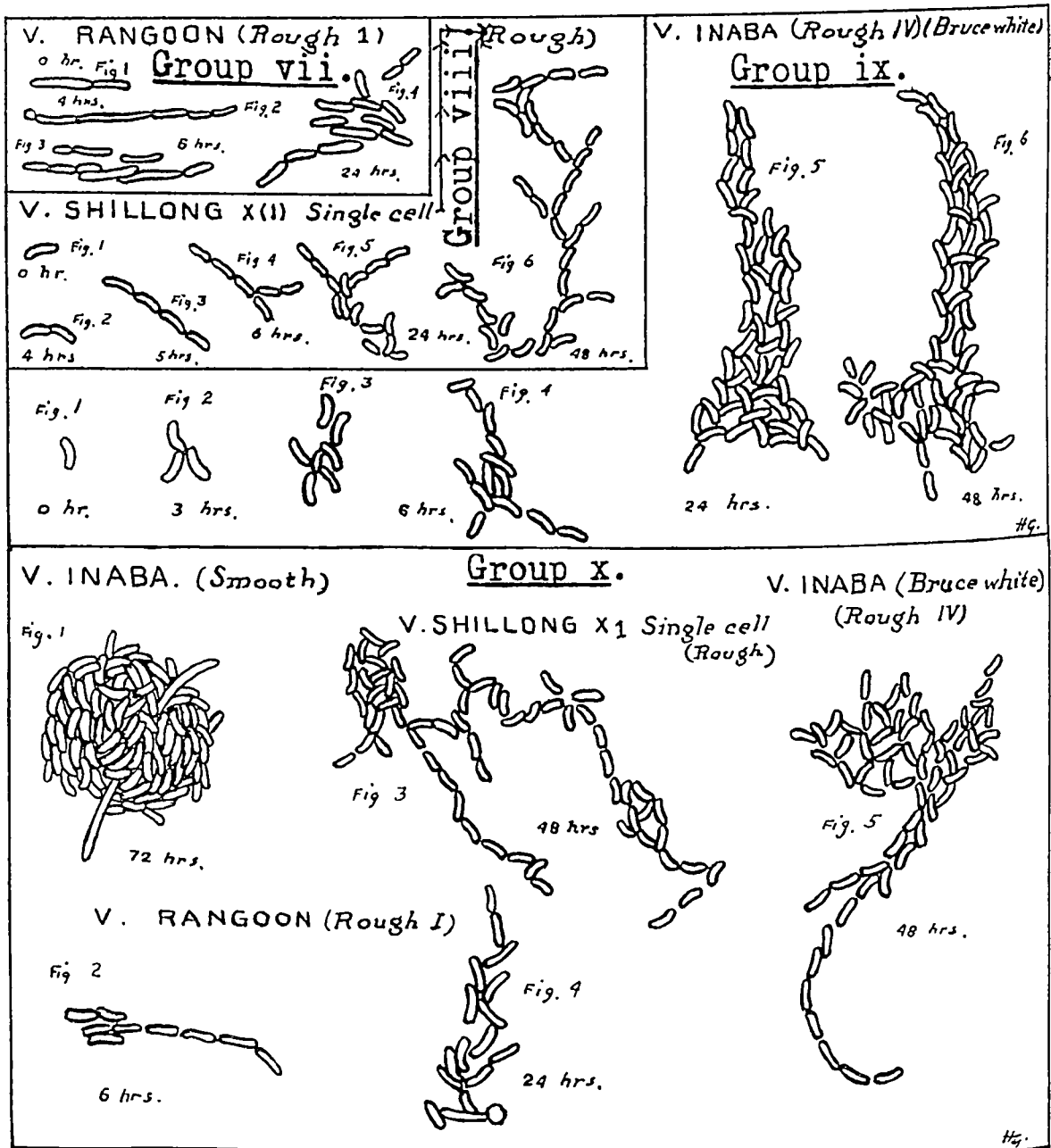


Fig. h.—A typical 'medusa-head' rough colony.

PLATE XXXVIII.



Groups vii to ix—showing the various stages of growth of *V. Rangoon rough I* (group vii), *V. Shillong X(1) single cell rough* (group viii) and *V. Inaba rough IV*—Bruce White (group ix), starting from a single cell in each case and sketched at intervals usually of 2 to 48 hours.

Group x—showing some extra sketches of *V. Inaba smooth* (72 hours), *V. Shillong X(1) single cell rough* (48 hours), *V. Inaba rough IV*—Bruce White (48 hours) and *V. Rangoon rough I* (6 hours and 24 hours).

Preparations of rough cultures of these organisms were made and studied in the same way as the smooth ones described above. Isolated viable organisms were singled out and watched under the darkground illumination. There was the same lag period as in the case of smooth cultures but it was slightly more prolonged. In fact, when the number of organisms was counted after a certain interval, say, 24 hours, it was found to be only about half those in the smooth culture.

On watching a single cell of the rough culture under the darkground illumination, generally a refractile granular-looking material is found in the cell-cytoplasm, the cell outline is slightly thicker and the cell as a whole looks somewhat more opaque and refractile than a smooth one. A definite distinction can also be found in the microphotographs of the rough cultures, in that they look more opaque and in most instances the walls of the individual cells, especially at their ends, look thicker than those of the smooth ones (Plate XXXIII, figs. 8 to 20). After the organisms have divided once more they tend to bend at an angle (Plate XXXIII, figs. 11 and 12) and often form long chains which may be straight, bent at one or several places (Plate XXXIII, figs. 13, 14, 15 and 19), or even sometimes branching (Plate XXXIII, figs. 17 and 19; Plate XXXVII, group vi, figs. 7 and 8). These chains are composed of either a single row of organisms (Plate XXXIII, figs. 13, 14 and 19; Plate XXXVII, group v, fig. 5, and Plate XXXVIII, group vii, fig. 2) or of several rows forming compound chains (Plate XXXIII, figs. 15 to 17; Plate XXXVII, group vi, fig. 8). The tendency to slip past each other is very weakly developed or almost absent in the case of the rough type. And although some of the organisms in a rough culture may show a tendency to slide and an organism which starts by dividing in the rough manner may show in a few divisions the sliding type, yet on looking at the growth as a whole it is found very irregular and can be easily distinguished from that obtained in a smooth culture (Plate XXXVII, group vi, figs. 6 and 8). The rough vibrios after division remain more adherent to their parents than in the latter resulting in the formation of chains which bend and branch and thereby give rise to the rough granular growth. In 24 hours, instead of forming a compact and regular cluster of cells like the smooth culture, they spread irregularly leaving spaces inside the cluster and often tailing off into branches or chains (Plate XXXIII, fig. 19; Plate XXXVII, group vi, fig. 8, and Plate XXXVIII, group ix, figs. 5 and 6).

Sometimes one or two of the daughter cells separate from the parent body of cells which have undergone only several subdivisions and instead of clinging to the latter as in the smooth culture tend to move away from it to form small daughter colonies. Thus the entire macroscopic colony is sometimes formed by the agglomeration of several contiguous small clusters of cells intermingled with and spread irregularly around the original one and looking like flowers under darkground illumination (Plate XXXIII, fig. 18).

Another characteristic of the growth of the rough type is that not only are the cells piled up above the surface of the medium but also there is a tendency to burrow deep into its substance. This tendency was not noted in smooth cultures in which long chains do not occur. This may be the reason why the rough colonies on agar medium sometimes adhere more firmly to it and are difficult to take off with a loop.

The irregular manner, described above, in which the rough type of vibrios divide and multiply, is related to the rugose and irregular appearance of the colonies. Also, as each individual vibrio with its wall and cytoplasm is more opaque than that of the smooth one, the rough colony as a whole looks more opaque than the latter which is usually clear and translucent.

The figures in Plates XXXVII and XXXVIII, groups v to ix, are the sketches of the organisms at various stages of their growth, made with the help of camera lucida. Microphotographs of some of them have also been taken under similar conditions and are shown in Plate XXXIII, figs. 8 to 19, demonstrating the actual mode of division and growth of rough vibrio strains starting from a single cell.

### (3) MIXTURES OF SMOOTH AND ROUGH CULTURES.

The following artificial mixtures were used to study the characters of mixed cultures :—

(1) V. INABA (smooth) + X(1) single cell ' R ' (rough).

(2) V. 10404 (smooth) + 1617 single cell ' R ' (rough).

Preparations of the above mixed cultures were made and observed as in the pure smooth and rough cultures. In each case both the smooth sliding type and the irregular chain type of growths were noted side by side in the same field, the number of each type varying according to the proportion of the mixed cells taken off with the loop to prepare the slide. The mixed cultures were then plated and typical smooth and rough colonies were picked out separately and their individual growth was watched in the similar manner. The latter, however, conformed to the usual character of the type selected, that is, cultures from the smooth colonies showed the smooth sliding type and those from the rough ones showed the irregular chain type of growth.

The so-called smooth culture of *Vibrio cholerae* may sometimes be composed mainly of smooth elements with a certain proportion of rough elements and an apparently rough culture may be slightly mixed with the smooth (Plate XXXIV, figs. b, c and d; Plate XXXV, fig. g, and Plate XXXVI, figs. j, k and l). Therefore, while watching the growth under the darkground illumination, if one comes across a single cell of the rough character in an apparently smooth culture or vice versa, he may perhaps consider the smooth as rough and the rough as smooth or conclude that both ' S ' and ' R ' types divide and multiply practically in the same manner. But on looking through the whole slide carefully it will be found that most of the growths in other fields conform to the usual character of either smooth or rough type according to the one selected for study. This difficulty can be cleared by plating the test culture and picking out the smooth and rough ones separately and then watching their individual growth in the manner described in the case of the mixed culture.

### DISCUSSION.

The appearances observed under the darkground illumination during the multiplication of the organism on a soft solid medium strongly suggest that the process of cell division and colony formation are distinct for smooth and rough types of *V. cholerae*. The individual cell of the rough type has a more opaque and

granular cytoplasm and a thicker outline than that of the smooth one which, on the other hand, possesses a clear cytoplasm and a thin wall. This may explain why the rough colonies usually look opaque and the smooth ones clear and translucent to the naked eye. Also, once the division starts the cells of the smooth type multiply more rapidly than those of the rough type, so that the cluster formed by the former after a certain interval of time is definitely larger and contains more cells than that of the latter. The essential difference between the rough and smooth types, however, depends upon the degree to which the contiguous cells adhere to each other after undergoing division. The final cluster in a smooth culture is even in appearance owing to the cells sliding past each other and forming smooth and compact masses, while in case of rough cultures the cells usually form chains which bend at angles at several places or form compound or branching chains and irregular masses; the tendency to slip past each other is very weakly developed in some and practically absent in others and often the whole mass is but an agglomeration of several scattered and interconnected groups of cells with many open spaces left in between; many projections, angles, and sometimes chains, stick out from the side of the irregular mass. All these considered together are at the basis of the granular and rugose appearance of the colonies formed by the rough type. Again, it is usually observed that a rough colony on an agar medium can be easily pushed along by means of a loop and remain intact, while the smooth colony will break up at a touch. This difference is perhaps due to the fact that the irregular masses of cells composing the rough colony are actually intermingled and bound together by means of chains and irregular offshoots. While in the smooth culture, although the cells are arranged in a regular and compact manner, they are not so firmly adherent or intertwined with one another as in the rough culture.

Another characteristic of the rough culture is its tendency to send rows of cells into the substance of the medium in addition to piling up on its surface. It may be on account of this behaviour that sometimes the rough colonies seem to adhere more firmly to the medium and are difficult to take off with a loop.

Some of the organisms in a rough culture observed showed a certain amount of mixed character, that is, they started by dividing in a rough manner and then for a few subsequent divisions they showed the sliding type of division (Plate XXXVII, group vi, figs. 6 and 8). But an organism in a single cell rough culture has never been observed to divide throughout in a typical smooth way. The final cluster in such a case is of the jagged uneven appearance of the rough type and is therefore sharply distinguishable from that of the regular smooth culture. The above behaviour may be either one of the general mode of division in a rough culture or only an indication of a preliminary stage of forming a smooth dissociant which is sometimes found in a subculture of a rough type of *V. cholerae* (Plate XXXV, fig. f; Plate XXXVI, figs. k and l). On the other hand, in none of the smooth cultures was the irregular bending division observed. It should be borne in mind, however, that an apparently rough or smooth culture may be a mixture of both, and in that case the entire slide will show a mixture of smooth and rough types of cluster and that a rough dissociant in a smooth culture is just as possible as a smooth dissociant in a rough culture as stated above (Plate XXXVI, fig. j).

The rough and smooth vibrio cultures behave similarly in the liquid (broth or peptone water) as in the solid medium described above. The rough strains form chains leading to the development of irregular clusters which form a thick pellicle on the top as well as granules which either fall to the bottom leaving the fluid almost clear or remain partially suspended in it producing slight turbidity. The smooth strain, on the other hand, forms a uniform and much thicker turbidity with or without a thin pellicle in the same media due to the even suspension of the organisms which separate or slide past each other and do not stick together after division or fall to the bottom.

Thus, in respect of division and multiplication from a single cell, both the smooth and rough cultures correspond exactly to those of the smooth or rough types of the bacilli of the *Salmonella* group as shown by Nutt (*loc. cit.*). Perhaps other bacteria also follow the same principle in relation to the smooth and rough colonies and the phenomenon may be considered as a general one. We have, therefore, in this study found another means of differentiating the smooth from the rough type of *V. cholerae*.

#### SUMMARY.

1. A method for observing the growth of vibrios from a single cell under the darkground illumination has been described. A combination of 0.5 per cent agar and 12 to 15 per cent gelatin forms a satisfactory medium for watching the growth of the rough and smooth types.

2. A study of the rough and smooth cultures of *V. cholerae* undertaken along the above lines definitely shows that the process of cell division and colony formation are distinct for each of them. The essential difference depends upon the degree to which the contiguous cells adhere to each other after undergoing division. The final cluster in a smooth culture is even in appearance owing to the cells sliding past each other and forming a smooth and compact mass, while in a rough culture the tendency to slip past each other is almost absent and the cells after division tend to adhere to each other more firmly leading to the formation of bending and branching chains and irregular masses with many open spaces, projections, angles, and sometimes chains, sticking out from their sides, the final cluster being thus jagged and uneven in appearance.

3. A similar study of the mixtures of the rough and smooth cultures of the above organism indicates that an apparently smooth or rough culture may be a mixture of both, only varying in proportion, and that the change from the smooth to rough is a gradual process particularly in regard to the colony appearances.

4. The above observations have been supplemented by camera-lucida drawings and, wherever possible, by microphotographic illustrations.

5. It has also been noted that in the case of *V. cholerae* the causes that lead to the formation of rough colonies on solid media operate equally in broth and peptone water.

6. An attempt has been made to explain the various characters of the rough and smooth colonies on the basis of the above findings.



## ACKNOWLEDGMENTS.

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## BIOLOGICAL VALUE OF THE PROTEINS OF SOYA BEAN, FIELD PEA, AND *LATHYRUS SATIVA*.

BY THE BALANCE-SHEET AND GROWTH METHODS.

BY

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LEGUMINOUS seeds form the most important source of proteins for the majority of the Indians. Two of the common pulses, green gram and lentil, have already been investigated in these Laboratories (Basu, Nath and Ghani, 1936*a* and *b*). The determination of the nutritive value of the proteins of soya bean, field pea (*Pisum sativum*, matar), and *Lathyrus sativa* (khesari), both by the balance-sheet and growth methods, forms the subject-matter of this investigation.

Soya bean, because of its high protein content, deserves to be popularized in this country, especially in Bengal where the diets are deficient both with regard to the quantity and the quality of proteins. The variety selected for this investigation was pure-line strain of the yellow Barmali variety grown in the Agricultural Farm at Darjeeling. This was kindly supplied by Mr. D. Dutt, Economic Botanist to the Government of Bengal. Mr. Dutt has been able to raise this crop successfully on the Dacca soil.

*Lathyrus sativa* is largely consumed in India. When on account of the failure of the rains and resulting dryness of the soil, the wheat crop fails, *Lathyrus sativa* (khesari dal) forms the main and practically the only staple food for the poor

people in some parts of India like Rewa. It is supposed to induce lathyrism which is generally a phenomenon of famine years when the diet is wholly made up of khesari dal.

Field pea (*Pisum sativum*) is also largely taken in India. In bazaar samples *Lathyrus sativa* and field pea are generally mixed. The samples were, therefore, very carefully sorted out by recognition and we worked with practically pure samples of field pea and *Lathyrus sativa*.

## I. THE BALANCE-SHEET METHOD.

### *Technique.*

The technique employed was the same as in the previous investigation from these Laboratories (Basu *et al.*, 1936*a*). Instead of using two adult rats in each metabolism cage only one male rat, weighing between 160 g. and 315 g., was placed in each cage. In experiments with nitrogen-free ration there was a preliminary period of three days, the collection of the faeces and urine was made for the subsequent five days. With protein diets there was a pre-experimental period of four days and a collection period of five days. Both in the experiments of Basu *et al.* (*loc. cit.*) and in these and other similar investigations from these Laboratories the rat or rats were kept in the metabolism cage during the preliminary period so that they might get accustomed to the experimental conditions and take sufficient food during the collection period.

Schneider (1934) has expressed some doubt whether this preliminary period of three or four days and a collection period of four or five days are sufficient to get rid of the effect of previous diet. The whole question has recently been examined by Chick, Hutchinson and Jackson (1935*a*) and they find that the preparatory period of two to three days and a collection period of four days as employed by that school were quite sufficient. In our experiments an interval of one week was allowed between two consecutive experiments with the same set of rats, during which period they were given the whole-wheat and milk ration, and kept in ordinary cages in which there was free access of light and air. This was also done by Basu *et al.* (*loc. cit.*).

An essential condition for the success of the method is that dietary proteins should not be utilized as a source of energy and hence the results were accepted only of those experiments in which the food intake and hence the intake of non-protein calories were sufficient (not less than 120 kg. calories per day per kg. of body-weight). Vitamin-B complex was supplied in the form of an active yeast preparation.

### *Metabolic nitrogen of the faeces.*

The workers at the Lister Institute (Chick *et al.*, 1935*a*) have re-investigated this problem and contrary to their previous observations their present results support Schneider's (*loc. cit.*) view that metabolic nitrogen varies with food intake and depends on body-weight. They also found that faecal endogenous nitrogen excretion showed a great constancy in experiments of similar duration. The metabolic nitrogen in the present case has been calculated on the basis of Schneider's

principle. In using Schneider's principle, the points from the data from nitrogen-free experiments were plotted in two separate groups in two figures—one for rats weighing more than 250 g. and the other for rats weighing less than 250 g.

*The endogenous nitrogen of urinary origin.*

Ashworth and Brody (1933) found that endogenous urinary nitrogen per kg. body-weight for rats on a nitrogen-free diet did not reach a minimum value until 10 to 15 days after the diet was given. Rochee (1933) also obtained similar results. Mitchell (1924) had observed a very rapid fall in endogenous urinary nitrogen on the first day of the nitrogen-free diet and then found it to remain roughly constant. Chick *et al.* (1935a) found a rapid fall in urinary nitrogen during the first two to three days on a nitrogen-free diet and subsequently a very gradual decrease. Thus the preliminary period of three days, which elapsed before our experiment proper was made, was long enough to include the initial rapid fall in urinary nitrogen excretion. This initial drop in the urinary nitrogen excretion also occurs on low protein diets and hence cannot greatly affect the calculation of biological values.

*Materials used.*

Table I gives the composition of the cereals used as sources of protein. Table II gives the composition of the diets used in these experiments. Table III represents the results of experiments with nitrogen-free diet. Results of metabolism experiments with soya bean, field pea, and *Lathyrus sativa* are given in Table IV.

The same expression has been employed for calculating the biological value, as was previously done by Basu *et al.* (1936a). Recently the Lister school (Chick *et al.*, 1935a) have given up their old formula and adopted Mitchell's expression for calculating biological values.

TABLE I.

*Analysis of pulses used as sources of protein.*

Pulse.	Moisture (per cent).	Total nitrogen (per cent).	Protein N $\times$ 6.25 (per cent).	Ether extractives (per cent).	Ash (per cent).	Crude fibre (per cent).	Carbo- hydrate (by diff.).
Soya bean ..	11.5	6.59	41.2	17.5	4.1	4.2	21.5
Field pea ..	13.5	4.34	27.1	2.1	3.2	0.8	46.7
<i>Lathyrus sativa</i>	12.1	5.15	32.2	2.0	2.1	0.9	49.8

TABLE II.

*Composition of diet.*

Diets.	Nitrogen free.	Soya bean (g.).			Field pea (g.).		<i>Lathyrus sativa</i> (g.).		Soya bean and wheat (1:2) (g.).	Field pea and wheat (1:2) (g.).		Field pea and <i>Lathyrus sativa</i> (1:1) (g.).	
		5 per cent	10 per cent	15 per cent	10 per cent	15 per cent	10 per cent	15 per cent		10 per cent	15 per cent	10 per cent	15 per cent
Protein ..	..												
Soya bean ..	..	122	243	366	..	..	..	..	80	..	..	..	..
Field pea ..	..	..	..	..	370	555	..	..	..	135	185	277	277
<i>Lathyrus sativa</i> ..	..	..	..	..	..	..	321	482	..	..	160	241	241
Wheat ..	..	..	..	..	..	..	..	..	576	732	..	..	..
Chopped sugar ..	90	90	90	90	90	90	90	90	90	55	90	90	90
Ghee ..	100	100	90	80	100	100	100	100	100	100	100	100	100
Cod-liver oil ..	20	20	20	20	20	20	20	20	20	20	20	20	20
Salt mixture ..	50	50	50	50	50	50	50	50	50	50	50	50	50
Calcium carbonate.	8	8	8	8	8	8	8	8	8	8	8	8	8
Corn starch ..	735	610	499	386	362	177	411	248	79	..	387	214	214
Total non-protein calorific intake (per gramme of diet).	5	4.08	3.92	3.45	3.65	3.27	3.75	3.43	4.59	4.40	3.71	3.79	3.79

TABLE III.

*Experiments with nitrogen-free ration.*

Date of commencement.	Rat number.	Average body-weight (g.).	Average change in body-weight (g.).	Food intake (g.).	Urine nitrogen (mg.).	Metabolic nitrogen (mg.).	Used for balance-sheet experiments with
1-1-36 ..	11	315	-3.2	12.8	63.76	24.9	10 per cent pea and 15 per cent <i>Lathyrus</i> diets.
25-12-35 ..	42	335	-2.7	15.2	59.97	28.7	15 per cent pea, 10 per cent <i>Lathyrus</i> and 10 per cent mixed diets.
1-1-36 ..	52	275	-2.1	12.7	40.39	22.5	15 per cent <i>Lathyrus</i> diet.
25-12-35 ..	72	253	-3.2	12.2	52.92	22.6	15 per cent pea diet.
25-12-35 ..	140	190	-1.9	11.2	38.89	19.69	10 and 15 per cent pea, 10 per cent <i>Lathyrus</i> and 10 per cent mixed diets.
19-12-35 ..	169	180	-1.7	7.0	49.03	16.45	15 per cent pea, 10 per cent <i>Lathyrus</i> and 15 and 10 per cent mixed diets.
1-1-36 ..	179	190	-1.8	10.3	33.25	18.82	10 per cent pea and 15 per cent mixed diets.
30-1-36 ..	196	176	-1.5	9.4	31.34	18.18	15 per cent mixed diet.
11-4-36 ..	140	209	-2.0	6.0	35.00	12.00	15 and 5 per cent soya bean, 10 per cent cooked soya bean and 10 per cent pea-wheat diets.
19-4-36 ..	159	234	-2.0	5.2	29.72	12.85	5 per cent cooked soya bean and 10 per cent soya-wheat diets.
11-4-36 ..	179	228	-1.0	6.2	38.63	13.50	} 15 and 10 per cent soya bean and 10 per cent soya-wheat diets.
20-7-36 ..	179	246	-1.5	9.1	33.40	18.60	
19-4-36 ..	194	231	-1.4	7.8	34.22	16.31	} 10 per cent soya bean and 10 per cent pea-wheat diets.
29-7-36 ..	194	275	-2.0	6.3	35.34	13.15	
19-4-36 ..	195	215	-1.3	5.2	33.37	11.92	} 15 and 10 per cent soya bean, 15 per cent cooked soya bean and 10 per cent pea-wheat diets.
20-7-36 ..	195	250	-1.0	7.7	35.20	15.10	
19-4-36 ..	196	231	-0.0	5.0	34.44	14.13	} 10 per cent soya bean diet.
29-7-36 ..	196	293	-3.0	7.3	32.01	18.46	
19-4-36 ..	197	161	-0.7	6.5	30.17	15.67	} 15 and 5 per cent soya bean and 10 per cent pea-wheat diets.
20-7-36 ..	197	210	-1.5	5.2	31.22	13.49	

TABLE IV.

*Biological value of proteins of soya bean, field pea, and Lathyrus sativa.*

Protein content (per cent).	Rat number.	Average body-weight (g.).	Average change in body-weight (g.).	Food intake (g.).	Non-protein calorie intake per kg. body-weight.	NITROGEN INTAKE (MG.).		URINE NITROGEN (MG.).			FÆCAL NITROGEN (MG.).			Biological value.	Biological value (mean).
						Total.	True.	Total.	Endogenous.	True.	Total.	Endogenous.	Exogenous.		
<i>Soya bean.</i>															
5	140	210	1.0	9.4	140	77.1	65.77	57.96	35.0	22.96	29.43	18.1	11.33	65	64
5	179	225	1.2	8.7	162	71.3	59.25	57.32	36.0	21.32	29.25	17.2	12.05	63	
5	197	175	0.7	8.5	204	69.7	56.4	51.73	30.7	21.03	30.21	16.9	13.31	63	
5 (cooked)	159	250	0.9	12.1	203	99.2	91.35	73.19	29.7	43.49	29.44	21.6	7.84	52	52
10	194	238	1.0	8.9	146	130.8	114.19	80.14	34.8	45.34	34.01	17.4	16.61	60	
10	195	220	1.4	9.2	163	135.2	117.76	82.12	34.3	47.82	35.34	17.9	17.44	59	
10	196	245	1.3	10.5	167	154.3	127.14	88.87	33.2	55.67	46.66	19.5	27.16	56	58
10 (cooked)	140	250	0.0	8.2	122	124.3	111.5	81.28	35.0	46.28	29.4	16.6	12.8	50	
15	140	243	0.0	8.3	124	195.9	176.86	114.71	35.0	79.71	33.74	16.7	17.04	55	
15	179	247	1.3	9.7	130	228.9	206.7	127.52	36.0	91.52	45.74	18.5	27.24	54	54
15	195	237	0.5	7.9	120	186.4	172.52	116.54	34.3	82.15	30.18	16.2	13.98	56	
15	197	208	0.8	9.7	138	224.2	194.4	123.8	30.7	93.11	48.02	18.2	29.82	52	
15 (cooked)	195	248	-0.3	8.5	123	176.7	166.1	121.88	34.3	87.58	27.5	16.9	10.6	47	47



*Pisum sativum.*

10	11	313	1.0	15.6	179	247.6	227.1	182.6	63.8	118.8	51.2	30.7	20.5	48
10	140	205	0.0	10.3	181	165.2	150.9	117.4	38.9	78.5	33.5	19.2	14.3	48
10	179	185	1.0	12.9	251	204.6	185.0	128.3	33.3	95.0	41.6	22.0	19.6	48
15	42	335	2.0	11.5	123	278.5	241.3	200.4	60.0	140.4	59.2	22.0	37.2	42
15	72	252	0.5	11.1	145	269.7	244.4	200.9	52.9	147.9	46.7	21.4	25.3	39
15	140	182	0.3	9.6	174	231.5	209.0	157.9	38.9	119.0	40.8	18.3	22.5	43
15	169	170	1.3	12.6	244	305.4	273.3	206.1	49.0	157.1	53.9	21.8	32.1	42

*Lathyrus sativum.*

10	42	335	0.4	14.0	155	229.2	202.2	166.4	60.0	106.4	52.8	25.8	27.0	47
10	140	190	0.4	10.6	206	173.2	157.6	116.4	38.9	77.5	35.0	19.4	15.6	51
10	169	190	0.1	12.5	243	205.1	183.8	138.9	49.0	89.9	43.1	21.8	21.3	51
15	11	312	1.4	12.9	140	314.3	281.9	223.2	63.8	159.5	56.6	24.2	32.4	44
15	52	277	0.9	10.5	128	252.6	229.7	167.8	40.4	127.5	43.3	20.4	22.9	44

The digestibility and protein value of the different pulses are indicated in Table V:—

TABLE V.

*Digestibility and protein value.*

Protein in diet (per cent).	FOOD NITROGEN		Percentage digestibility.	Mean percentage digestibility.	Mean B. V.	Protein content of pulses (per cent).	Protein value.
	intake (mg.).	digested.					

<i>Soya bean.</i>								
5	77.1	65.77	85	}	83	64	41.2	21.7
5	71.3	59.25	83					
5	69.7	56.4	81					
5 (cooked)	99.2	91.36	92		92	52	41.2	19.3
10	130.80	114.19	87	}	85	58	41.2	20.2
10	135.2	117.76	87					
10	154.3	127.14	82					
10 (cooked)	124.3	111.5	90		90	50	41.2	18.5
15	195.9	178.86	91	}	89	54	41.2	19.7
15	228.9	206.7	88					
15	186.4	172.52	92					
15	224.2	194.4	87					
15 (cooked)	176.7	166.1	94		94	47	41.2	18.1

<i>Pisum sativum.</i>								
10	247.6	227.1	92	}	91	48	27.1	11.7
10	165.2	150.9	91					
10	204.6	185.0	90					
15	278.5	241.3	87	}	89	41	27.1	9.8
15	269.7	244.4	91					
15	231.5	209.0	90					
15	305.4	273.3	89					

TABLE V--*concl'd.*

Protein in diet (per cent).	FOOD NITROGEN		Percentage digestibility.	Mean percentage digestibility.	Mean B. V.	Protein content of pulses (per cent).	Protein value.
	intake (mg.).	digested.					
<i>Lathyrus sativa.</i>							
10	229.2	202.2	88	90	50	32.2	14.4
10	173.2	157.6	91				
10	205.1	183.8	90				
15	314.3	281.9	89	90	44	32.2	12.7
15	252.6	229.7	91				

*Effect of concentration of protein on the biological value.*

Table IV indicates that as the concentration of protein in the diet increases the biological value gradually decreases. This is brought out more clearly in the following table:—

TABLE VI.

Pulses.	MEAN BIOLOGICAL VALUES OF THE PULSES AT		
	5 per cent level.	10 per cent level.	15 per cent level.
Soya bean ..	64	58	54
<i>Pisum sativum</i> ..	..	48	41
<i>Lathyrus sativa</i> ..	..	50	44

Previous investigations in these Laboratories on the biological value of the proteins of green gram and of lentil (Basu *et al.*, *loc. cit.*) yielded similar results, the fall in biological values with increase in protein concentration being more marked there.

Boas-Fixsen (1930) found the biological value of heated purified caseinogen to be independent of its concentration in the diet. But later on Boas-Fixsen and Jackson (1932) stated that the biological value of proteins diminished with increase in concentration of protein. More recently Chick *et al.* (1935b), working at protein levels 3 to 10 per cent in the diet with whole wheat, white flour, wheat germ, maize endosperm, whole milk, lactalbumin, and caseinogen, found that, except in the case of whole-milk proteins the biological value of which remained uniformly high at all levels, increasing the proportion of protein in the diet lowered the biological value.

## II. THE GROWTH METHOD.

The experiments with the balance-sheet method show that for the maintenance of nitrogen equilibrium the proteins of soya beans are superior to those of the field pea and the *Lathyrus sativa*, while the two latter pulses are equally efficient in this respect. Considering the probability that they might behave differently in promoting growth, the growth-promoting capacity of these pulses has also been investigated.

*Technique.*

The same method was adopted as was done by Basu *et al.* (1936b) in their investigations on the green gram and the lentil. In growth experiments of this nature the provision of adequate amounts of vitamin-B complex without at the same time introducing considerable amounts of foreign protein is essential. As in the experiments of Basu *et al.* (1936b), vitamin-B complex was added to the diets in the form of 2 c.c. of a very active preparation of the yeast every day. Control experiments with rats on a diet, containing all the necessary ingredients except vitamin B and to which 2 c.c. of this yeast preparation were added every day, showed that this amount of vitamin B was sufficient for normal growth. The total amount of nitrogen given to the rats in the course of eight weeks from this source was 51.2 mg. The composition of the diets was the same as in Table II.

*Results.*

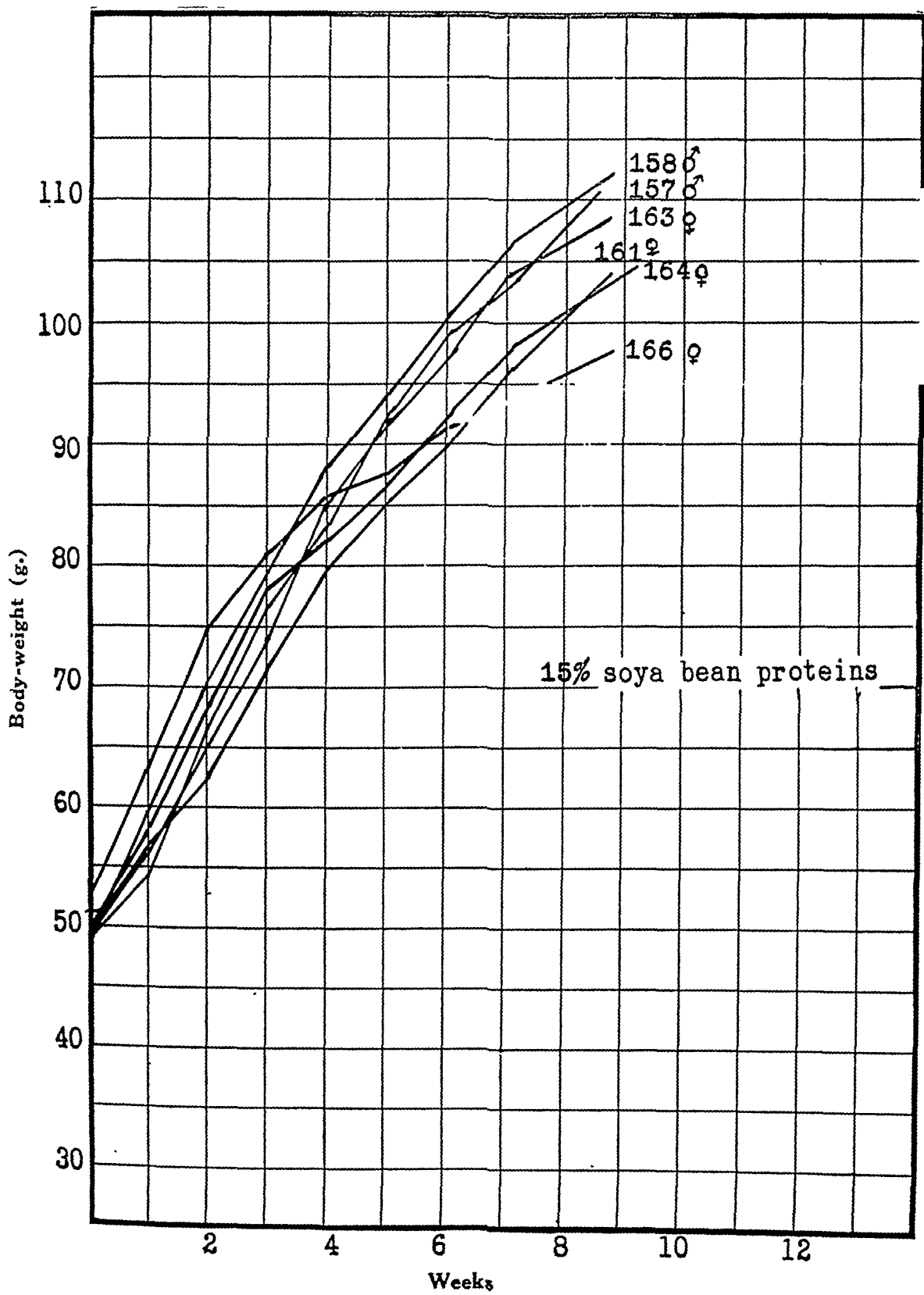
The performance of the individual rats is represented in Graphs 1 to 5. The results are summarized in Table VII. The biological values are calculated from the total protein intake and the corresponding gain in weight in four and in eight weeks.

*General condition of the rats on different diets.*

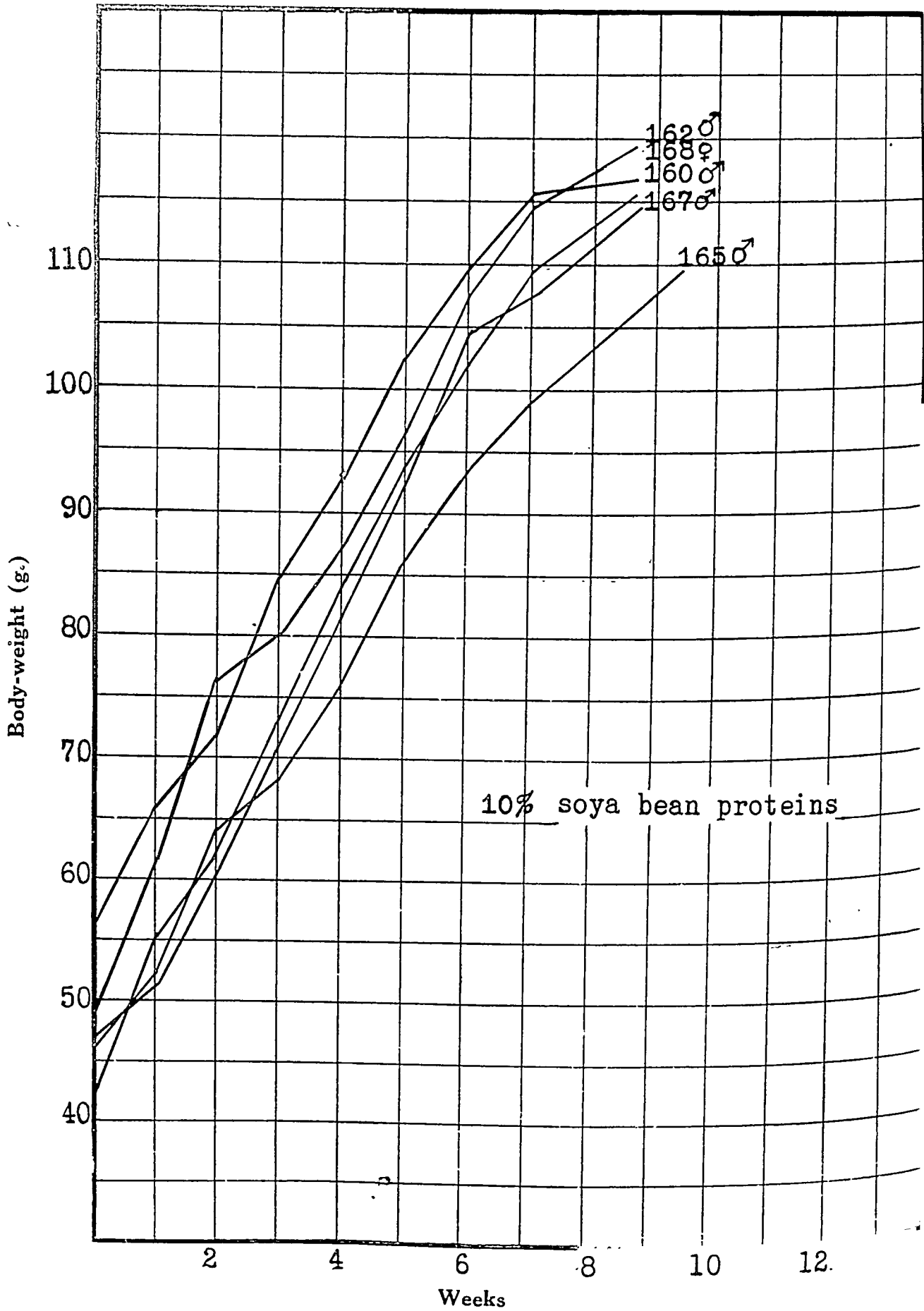
The animals receiving 15 per cent soya bean and 15 per cent field pea looked healthy. Loss of fur was noticed in rats receiving 10 per cent soya bean, 10 per cent field pea, and 15 per cent *Lathyrus sativa*, though it was not marked in the first two cases. The rats fed on 10 per cent *Lathyrus sativa* became much emaciated and looked very unhealthy; besides failure of growth, very marked loss of fur was also observed. Healthy appearance and silkiness and smoothness of fur were restored on adding a daily dose of 10 mg. of a tryptophane solution to the 10 per cent *Lathyrus sativa* diet, though no marked enhancement of growth was secured.

It would be seen from the figures and the table that when the growth of young rats is taken as the criterion, the proteins of soya bean are superior to those of field pea and *Lathyrus sativa*. Thus at 10 per cent of protein in the diet, the ratio  $\frac{\text{gain in weight}}{\text{protein consumed}}$  after eight weeks of feeding are 1.6 and 1.0 for the proteins of soya bean and field pea, respectively, and the rats fed on 10 per cent *Lathyrus* failed to grow. At 15 per cent concentration of protein the corresponding values for the proteins of soya bean, field pea, and *Lathyrus sativa* are 1.4, 0.9, and 0.6,

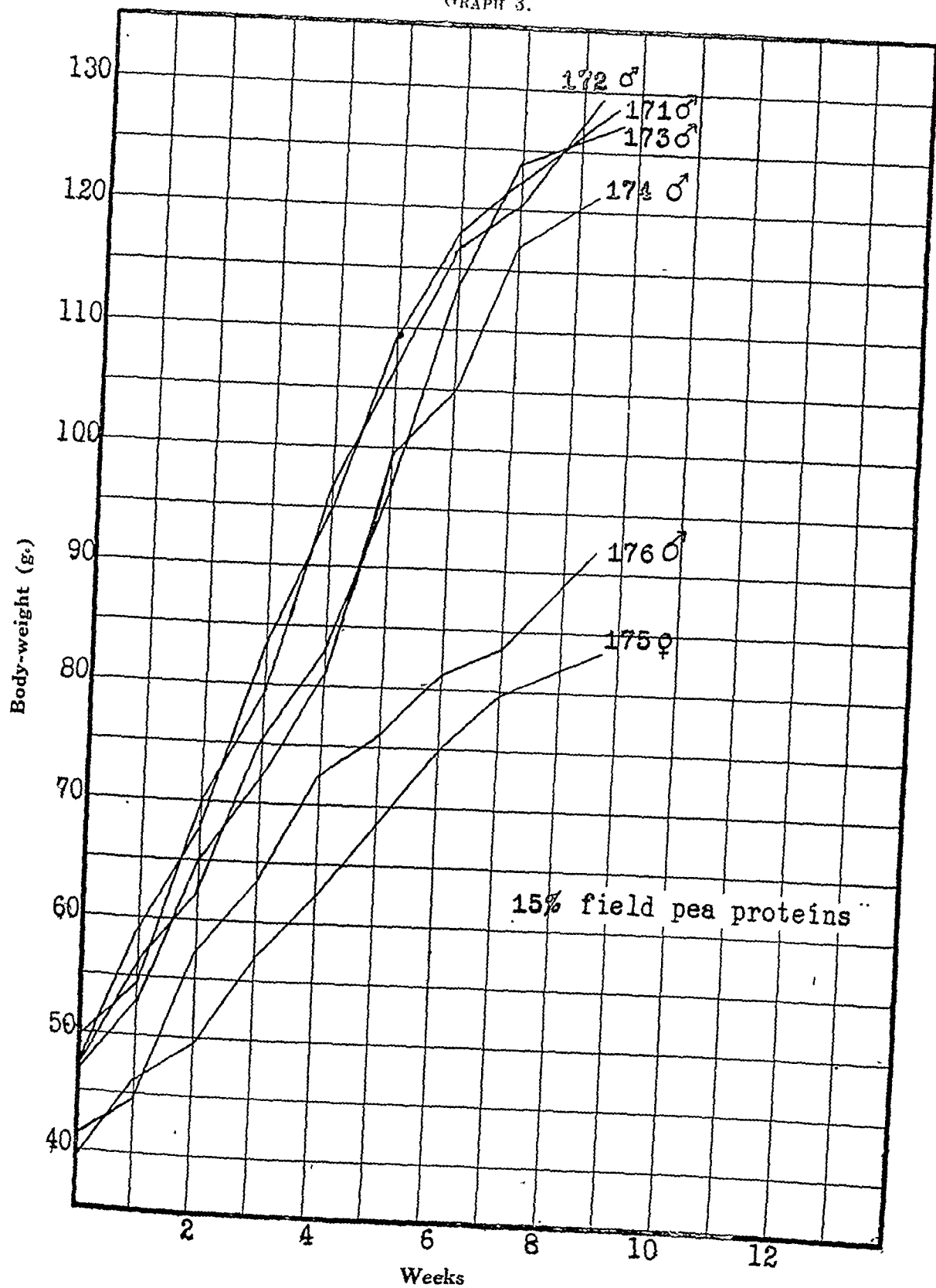
GRAPH 1.



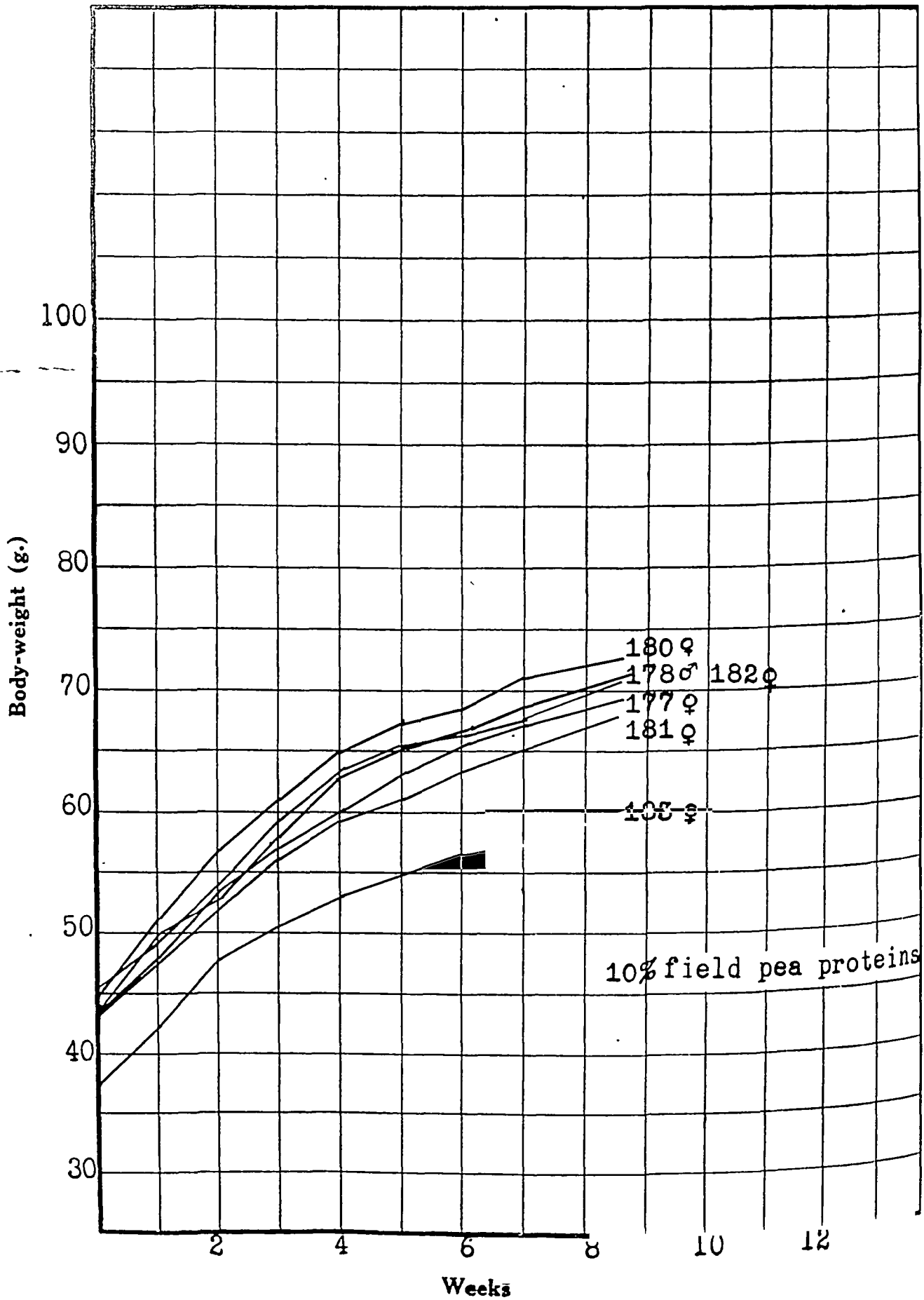
GRAPH 2.



GRAPH 3.



GRAPH 4.





GRAPH 5.

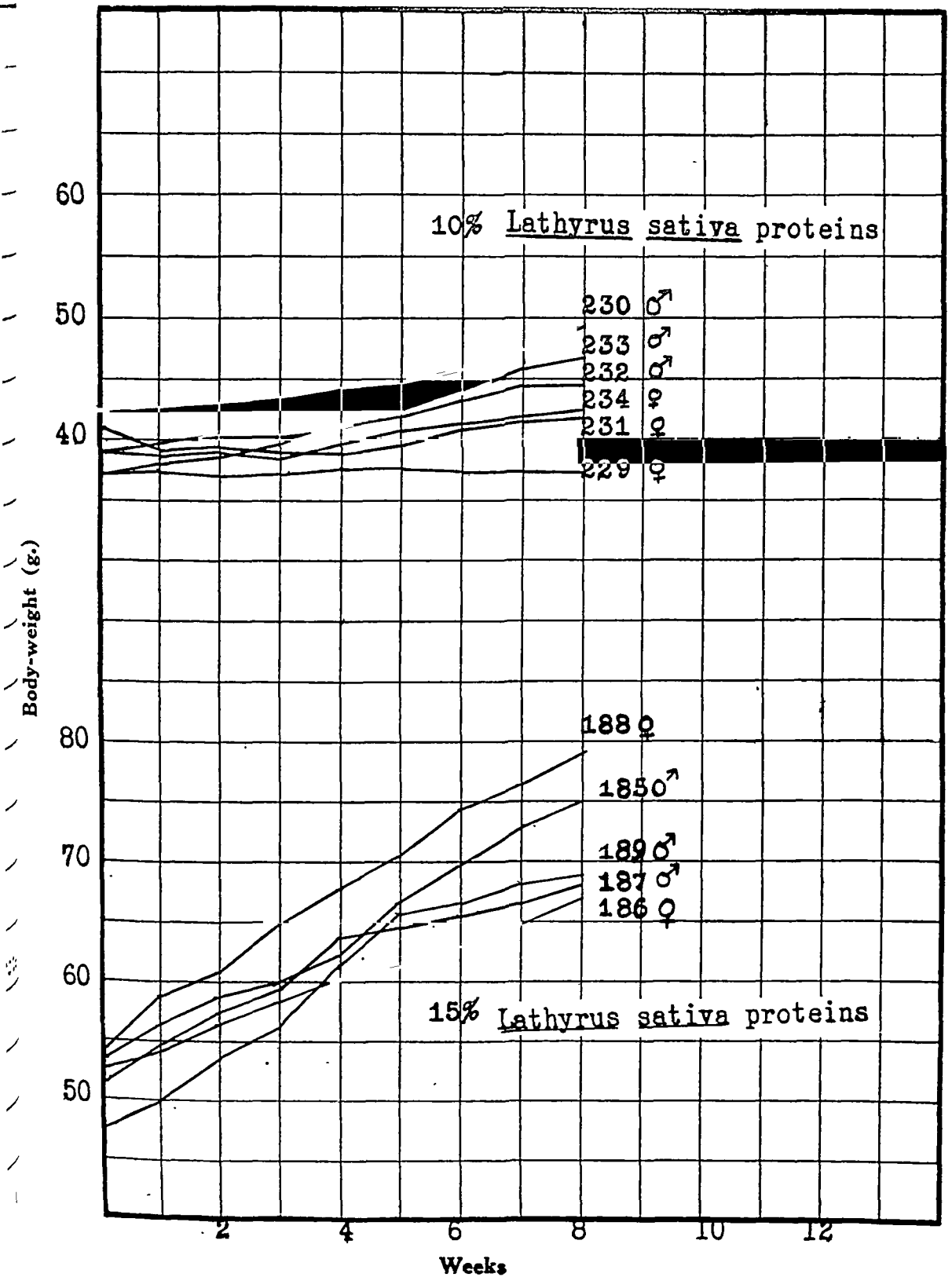


TABLE VII.

		FOUR WEEKS.				EIGHT WEEKS.						
Rat number.	Sex.	Initial weight (g.).	Food intake (g.).	Protein intake (g.).	Gain in weight (g.).	Gain in weight Protein intake = B. V.	Mean B. V.	Food intake (g.).	Protein intake (g.).	Gain in weight (g.).	Gain in weight protein intake = B. V.	Mean B. V.
15 per cent soya bean.												
157	M	49.5	124.2	18.6	33.7	1.8	1.9	272.7	40.9	58.5	1.4	1.4
158	M	49.0	126.0	18.9	38.9	2.1		280.0	42.0	60.6	1.4	
161	F	49.5	129.0	19.4	32.7	1.7		272.0	40.8	51.5	1.3	
163	F	49.0	125.3	18.8	36.0	1.9		269.3	40.4	57.5	1.4	
164	F	49.0	121.4	18.2	30.2	1.7		257.6	36.6	51.5	1.4	
166	F	52.9	126.9	19.0	33.0	1.7		262.5	39.4	43.5	1.1	
10 per cent soya bean.												
160	M	43.0	185.5	18.6	41.2	2.2	2.1	416.6	41.6	70.8	1.7	1.6
162	M	48.0	180.4	18.0	38.7	2.1		422.4	42.2	69.0	1.6	
165	M	46.0	157.4	15.7	29.7	1.9		362.6	36.3	57.0	1.6	
167	M	47.2	165.6	16.6	34.0	2.1		376.2	37.6	64.6	1.7	
168	F	56.0	183.8	18.4	36.8	2.0		405.2	40.5	59.9	1.5	

## 15 per cent Pisum sativum.

171	M	48.5	265.1	39.7	34.3	0.9	505.1	75.7	77.5	1.0
172	M	50.1	290.1	43.5	45.1	1.0	533.1	79.9	77.9	1.0
173	M	46.5	285.1	42.7	47.4	1.1	530.6	79.6	70.5	1.0
174	M	46.5	268.2	40.2	34.8	0.9	447.2	67.0	73.5	1.1
175	F	40.0	121.1	18.2	22.5	1.2	253.6	38.0	42.3	1.1
176	M	41.0	134.7	20.2	31.7	1.5	278.2	41.7	48.9	1.2

## 10 per cent Pisum sativum.

177	F	43.0	121.5	12.15	16.5	1.40	230.9	23.1	24.9	1.1
178	M	43.0	127.0	12.7	20.0	1.6	246.6	24.6	26.4	1.1
180	F	45.0	135.7	13.57	19.6	1.4	246.2	24.6	26.2	1.1
181	F	43.0	120.0	12.0	16.2	1.3	231.2	23.1	23.5	1.0
182	M	46.0	121.9	12.2	16.7	1.4	227.9	22.8	23.5	1.0
183	F	37.0	115.9	11.6	15.7	1.4	227.6	22.7	21.5	0.9

## 15 per cent Lathyrus sativa.

184	M	52.1	93.0	13.95	10.0	0.7	218.5	31.7	16.67	0.5
185	M	52.0	97.0	14.5	11.2	0.8	229.8	34.5	22.8	0.7
187	F	51.0	92.0	13.8	8.7	0.6	206.4	30.9	15.5	0.5
188	M	40.8	92.5	13.9	13.3	0.9	216.0	32.4	22.4	0.7
189	F	52.5	105.0	15.7	14.6	0.9	229.5	34.4	25.5	0.7
190	M	47.8	88.5	12.8	14.0	1.1	210.0	31.5	21.4	0.7

TABLE VII—concl'd.

Rat number.	Sex.	FOUR WEEKS.					EIGHT WEEKS.					
		Initial weight (g.).	Food intake (g.).	Protein intake (g.).	Gain in weight (g.).	Gain in weight $\div$ protein intake $\times$ B. V.	Mean B. V.	Food intake (g.).	Protein intake (g.).	Gain in weight (g.).	Gain in weight $\div$ protein intake $\times$ B. V.	Mean B. V.
10 per cent Lathyrus sativa.												
229	F	36.8	64.2	6.4	0	..	..	103.2	10.3	0	..	..
230	M	43.4	78.3	7.8	0.7	..	..	138.4	13.8	5.8	..	..
231	F	41.2	68.1	6.8	0	..	..	117.1	11.7	0.7	..	..
232	M	39.0	76.1	7.6	3.0	..	..	128.2	12.8	5.6	..	..
233	M	36.7	85.4	8.5	4.6	..	..	168.3	16.8	10.4	..	..
234	F	39.0	62.2	6.2	0.9	..	..	108.4	10.8	3.3	..	..

respectively. The superiority of the proteins of soya bean is also evident from the nature of the feeding curves. With only 5 per cent of protein in the diet, the gain in weight per gramme of soya protein is 0.6. The beneficial effect of tryptophane on general health and betterment of the condition of the hair of rats on *Lathyrus sativa* is in agreement with the observation of other workers (Alcock, 1936).

*Effect of the concentration of protein in the diet on the*  
ratio  $\frac{\text{gain in weight}}{\text{protein consumed}}$ .

It will be seen from the tables that for the same pulse the concentration of protein has not any marked effect on the value of the ratio.

*The maintenance requirement for rats with soya bean, field pea, and*  
*Lathyrus sativa.*

By plotting the increases in weight of the rats against protein intake of each, the experimental points are found to be grouped together about a straight line which when produced backwards cuts the abscissa at some point. The amount of protein corresponding to this point represents the protein requirement for maintenance for eight weeks, of rats weighing from 50 g. to 80 g. in body-weight. Boas-Fixsen *et al.* (1934) have obtained 10 g. of protein as the maintenance requirement for similar rats for a period of nine weeks with whole wheat and maize as the sources of protein. In the present case, it is shown in Graph 6 that the maintenance requirement with soya bean, field pea, and *Lathyrus sativa* are in decreasing order of magnitude, 7.5 g. in case of soya bean, 8.8 g. in case of field pea, and 9.3 g. in case of *Lathyrus sativa*.

*The effect of duration of experiments on gain per gramme of protein.*

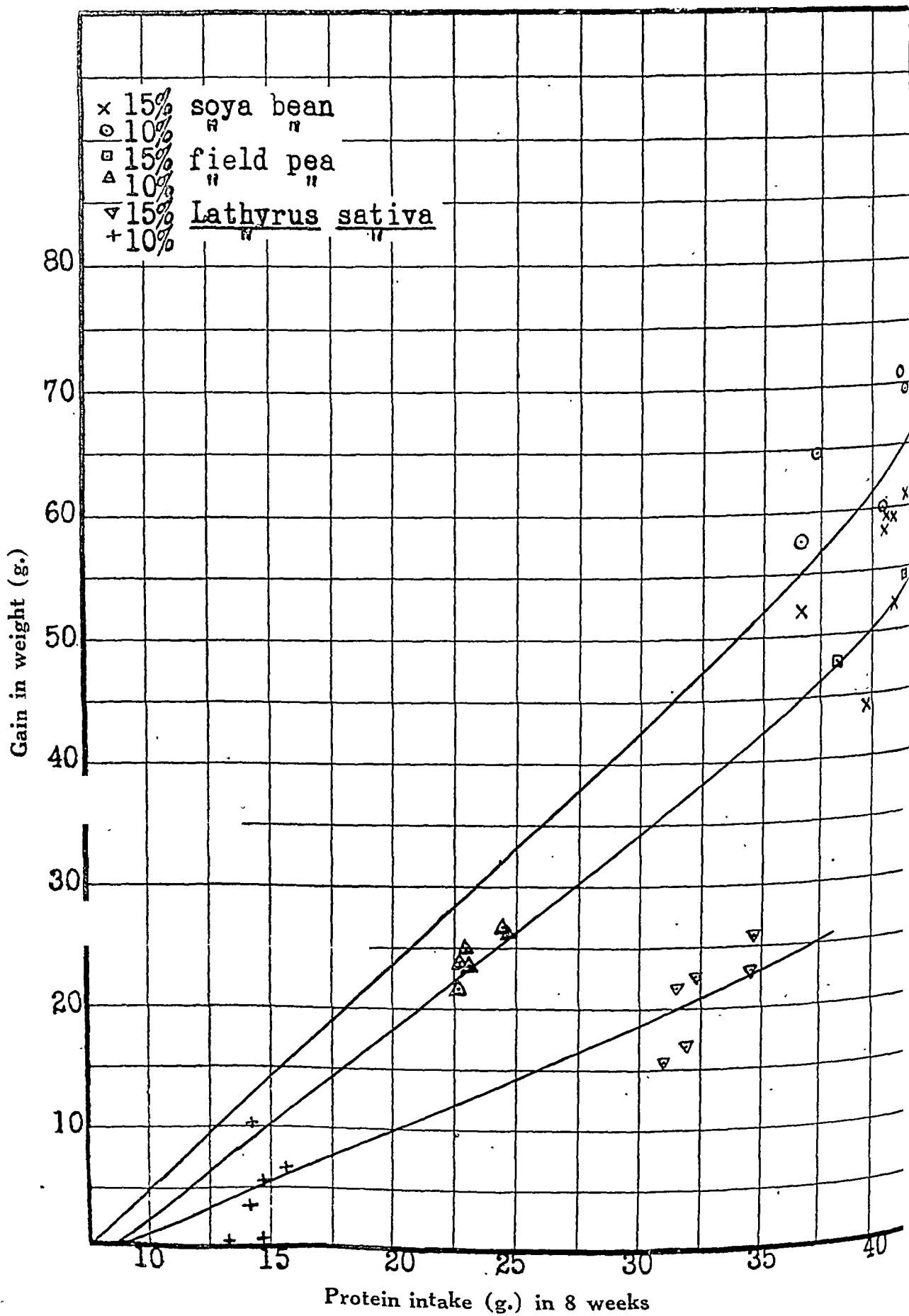
It will be seen from the table that as the period of experiment increases from four to eight weeks, the growth per gramme of protein diminishes. Similar results have been obtained by the previous investigators in this field (Osborne and Mendel, 1920; Basu *et al.*, 1936b).

*Supplementary relations.*

In growth experiments with rats a supplementary relation was found to exist between the proteins of soya bean and wheat by Kon and Markuze (1931) and between the proteins of pea and wheat bread by Markuze (1934). But we failed to obtain any supplementary relation between these proteins by the balance-sheet method. The biological value of wheat proteins was taken to be 61 at 10 per cent level, a value obtained by Boas-Fixsen and Jackson (*loc. cit.*). The results are indicated in Table VIII.

Experiments with mixed diets containing the proteins of pea and *Lathyrus sativa* in equal proportions showed definite negative results both by the balance-sheet and by the growth methods. The results by the balance-sheet method are given in Table VIII, while those by the growth method are given in Graph 7 and in Table IX.

GRAPH 6.



GRAPH 7.

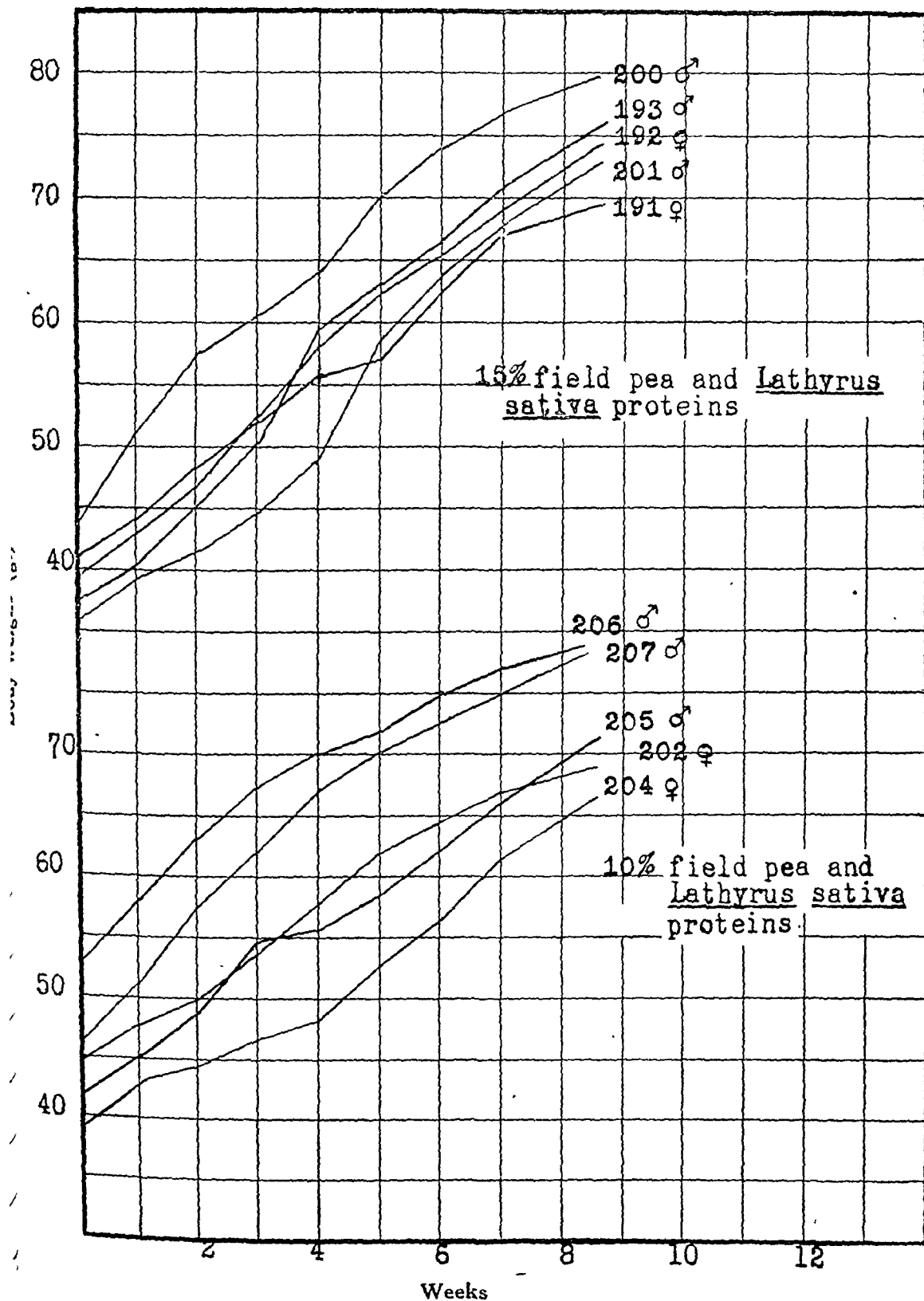


TABLE VIII.  
*Biological value and digestibility of mixed diets.*

Biological value and digestibility of

Protein (per cent).	Rat number.	Average body-weight (g.).	Average change in body-weight (g.).	Food intake (g.).	Non-protein caloric intake per kg. body-weight.	NITROGEN INTAKE, MG.		Digestibility.	URINARY NITROGEN.			FÆCAL NITROGEN.			B. V.	Mean B. V.	Calculated B. V.
						Total.	True.		Total (mg.).	Endogenous (mg.).	True (mg.).	Total (mg.).	Endogenous (mg.).	Exogenous (mg.).			
<i>Cooked soya bean + wheat proteins (1 : 2).</i>																	
10	159	280	1.0	9.6	157	150.7	137.74	91	84.09	29.7	54.39	31.26	18.3	12.96	61	60	57
	179	250	1.2	9.2	149	144.4	133.28	92	91.99	36.0	55.99	29.02	17.9	11.12	58		
	195	242	1.4	8.6	162	135.0	121.63	90	82.94	34.3	48.64	30.37	17.0	13.37	60		
<i>Pea + wheat proteins (1 : 2).</i>																	
10	140	248	0.5	8.5	150	133.4	120.96	91	84.98	35.0	49.98	29.34	16.9	12.44	59	60	57
	194	295	1.3	9.3	131	146.0	134.76	92	85.62	34.8	50.82	29.24	18.0	11.24	62		
	197	218	0.7	9.1	188	142.8	135.52	95	84.63	30.7	53.93	25.08	17.8	7.28	60		
<i>Pea + Lathyrus sativa proteins (1 : 1).</i>																	
10	42	334	0.4	14.5	160	230.6	211.1	91	166.8	60.0	10.9	46.2	26.7	19.5	49	48.5	49
	140	197	0.5	11.4	212	182.4	163.7	90	124.6	38.9	85.7	39.1	20.4	18.7	48		
	169	220	0.7	9.7	169	236.3	203.3	86	171.6	49.0	122.5	51.4	18.4	33.0	39		
15	179	203	1.2	11.9	222	289.3	263.3	91	194.8	33.3	161.6	47.0	21.0	26.0	39	40	42.5
	196	188	1.7	11.9	240	291.1	266.9	92	187.7	31.3	151.4	45.2	21.0	24.2	43		



TABLE IX.

Rat number.	Sex.	FOUR WEEKS.					EIGHT WEEKS.					
		Initial weight (g.).	Food intake (g.).	Protein intake (g.).	Gain in weight (g.).	Gain in weight = Protein intake = B. V.	Mean B. V.	Food intake (g.).	Protein intake (g.).	Gain in weight (g.).	B. V.	Mean B. V.
15 per cent mixture (pea— <i>Lathyrus sativa</i> proteins 1 : 1).												
190	M	41.2	95.5	14.3	18.8	1.3	1.2	197.6	29.55	35.0	1.2	1.1
191	F	39.2	92.5	13.9	17.3	1.2		207.8	31.17	28.8	0.9	
192	F	41.2	94.9	14.2	17.4	1.2		187.0	28.05	31.0	1.1	
193	M	37.9	95.5	14.3	21.1	1.5		204.3	30.64	36.3	1.2	
200	M	43.3	102.8	15.4	20.7	1.3		225.8	33.87	34.7	1.0	
201	F	35.8	95.3	14.3	13.0	0.9		214.3	32.14	35.2	1.1	
10 per cent mixture (pea— <i>Lathyrus sativa</i> proteins 1 : 1).												
202	F	44.3	135.8	13.6	11.2	0.8	1.1	307.9	30.8	23.7	0.8	0.8
204	F	39.0	132.0	13.2	9.5	0.7		292.7	29.3	26.0	0.9	
205	M	42.2	143.8	14.4	16.9	1.1		322.9	32.3	25.8	0.8	
206	M	53.0	141.7	14.2	16.5	1.1		322.6	32.3	23.8	0.7	
207	M	46.0	149.3	14.9	20.5	1.3		330.4	33.0	30.7	0.9	

*Lathyrism and Lathyrus sativa.*

In his report on lathyrism in the Central Provinces, Buchanan (1904) observed that lathyrism resulted in human beings when a diet of *Lathyrus sativa* of over 50 per cent mixture was taken for about three months. Acton and Chopra (1922) as a result of field and laboratory experiments arrived at the same conclusion. Howard, Simonson and Anderson (1923, 1925) carried out some experiments with ducks and monkeys, and observed that lathyrism was not induced by *Lathyrus sativa* but by the contaminating grain *Vicia sativa* (akri). McCombie Young (1927) suggested that lathyrism was caused by the lack of vitamin A. Acton and Chopra (1927) confirmed the results of Howard, Simonson and Anderson (*loc. cit.*) that the disease was caused by the grain *Vicia sativa*. McCarrison (1927) could not induce lathyrism in rats either with *Lathyrus sativa* (large or small seeded variety) or with pure akri (*Vicia sativa*) (*cf.* also McCarrison and Krishnan, 1934).

In our experiments, rats on a diet the protein of which was derived from *Lathyrus sativa*, but which was otherwise adequate, did not develop lathyrism. It is evident, therefore, that amino-acid make-up of the proteins of *Lathyrus sativa* is not responsible for lathyrism. We cannot, however, exclude the possibility that rats are immune from lathyrism.

## DISCUSSION.

In the balance-sheet experiments the soya bean was investigated in two ways: (i) the usual way as in the case of other pulses, and (ii) after making a paste with distilled water and cooking it in steam for three hours and subsequently drying in a current of air at 80°C. to 90°C. Tables IV and VI show that this prolonged cooking increases the digestibility but decreases the biological value of soya bean proteins. Thus at 5 per cent level, cooking increases the digestibility from 83 to 92 but decreases the biological value from 64 to 52. At 10 per cent level of protein, digestibility increases from 85 to 90 but biological value decreases from 58 to 50 on cooking. At 15 per cent level digestibility goes up from 89 to 94, while the biological value falls from 54 to 47.

Though the digestibility of the pulses *Pisum sativum* and *Lathyrus sativa* is higher than the corresponding values for soya bean, their biological values by the balance-sheet method are much lower. At 10 per cent level the biological values of *Pisum sativum* and *Lathyrus sativa* are 48 and 50, respectively, while at 15 per cent level the values are 41 and 44. It is also evident that the two pulses have the same biological value and are, therefore, equally efficient in maintaining nitrogen equilibrium.

It is evident that both as regards replacing waste tissues and formation of new ones, soya bean is much superior to field pea and *Lathyrus sativa*. Recently the use of soya bean in the dietary of human beings has been discouraged in some quarters. While soya bean can never replace milk, it has an advantage over other pulses in that it contains a higher percentage of proteins whose biological value is higher than that of many pulse proteins. Supplemented with small amounts of milk, soya bean should be a very useful article of foodstuff for the poor.

Although the field pea and *Lathyrus sativa* are equally efficient in replacing waste tissues, field pea is much superior to the *Lathyrus sativa* in promoting

growth. With the latter diet practically no growth is obtained at 10 per cent level of protein and the intake of food is very small. The intake scarcely exceeds the maintenance requirements. The poor performance of the rats on *Lathyrus sativa* diet is undoubtedly associated with low food intake. The loss of appetite is probably to be ascribed rather to some toxic factor present in *Lathyrus sativa* than to the amino-acid make-up of the proteins. This will be more fully dealt with in the next paper.

#### SUMMARY.

1. The biological values of soya bean proteins by the balance-sheet method at 5, 10, and 15 per cent levels of protein concentration are 64, 58, and 54, respectively, while those of the cooked product at the same levels are 52, 50, and 47. The values for field pea and *Lathyrus sativa* proteins at 10 and 15 per cent levels are 48, 41, and 50 and 44, respectively. The biological values thus decrease with increase in the concentration of protein.

2. The protein values of soya bean, cooked soya bean, field pea, and *Lathyrus sativa* are 20.2, 18.5, 11.7, and 14.4, respectively, at 10 per cent level.

3. Growth per gramme of protein ingested at 15 per cent protein concentration in the case of soya bean, field pea, and *Lathyrus sativa* is 1.4, 0.9, and 0.6, respectively. With 10 per cent protein the values in the case of soya bean and field pea are 1.6 and 1.0, respectively, while in the case of *Lathyrus sativa* practically no growth is obtained.

4. Rats on *Lathyrus sativa* diet took very little food but did not develop lathyrisms.

5. No supplementary relations could be observed between the proteins of wheat and of soya bean or field pea by the balance-sheet method. Field pea and *Lathyrus sativa* proteins showed no supplementary relations either by the balance-sheet or by the growth method.

6. The protein maintenance requirement for rats weighing from 40 g. to 80 g. appears to be the least in the case of soya bean proteins and greatest in the case of *Lathyrus sativa*.

#### ACKNOWLEDGMENTS.

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EXTRACTION AND CHEMICAL ANALYSIS OF THE  
PROTEINS OF GREEN GRAM (*PHASEOLUS*  
*MUNGO*), LENTIL (*LENS ESCULENTA*),  
AND *LATHYRUS SATIVA*  
(KHESARI).

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In previous investigations from these Laboratories (Basu, Nath and Ghani, 1936a and b), the biological values of the proteins of green gram and lentil have been determined and the green-gram proteins found superior to the lentil proteins by both the balance-sheet method and by the growth of young rats. Basu, Nath and Mukherjee (1937) have found that, though the proteins of field pea and *Lathyrus sativa* were equally efficient in maintaining nitrogenous equilibrium, the proteins of field pea were superior to those of *Lathyrus sativa* with respect to growth promotion. Indeed the rats fed on *Lathyrus sativa* diet at 10 per cent level of protein took very little food and showed only very slight growth; they appeared unhealthy and emaciated, and loss of fur was marked.

The cause of the difference in the biological value of the green-gram and the lentil proteins is to be sought in their amino-acid make-up. The behaviour of *Lathyrus sativa* proteins as opposed to field-pea proteins suggests that the former might be deficient in some essential amino acid or acids which are more necessary for growth promotion than for the maintenance of nitrogenous equilibrium.

The study of *Lathyrus sativa* proteins might also throw some light on the probable cause of the harmful effect of a prolonged diet containing this pulse which is said to induce lathyrism in human beings and in certain animals. It is to be noted that rats, kept on a *Lathyrus sativa* diet (10 per cent or 15 per cent protein) for a long time, were not found to develop any other abnormality except poor growth, general ill health, and loss of fur. McCarrison and Krishnan (1934) were also unable to induce lathyrism in rats by feeding them on a *Lathyrus sativa* diet. Field-pea proteins have been extracted and analysed by Osborne *et al.* (1907, 1908*a* and *b*).

#### CHEMICAL COMPOSITION OF THE PULSES.

The air-dried pulses were decorticated and the seeds after removal of the seed coats were ground to a fine powder and allowed to pass through a sieve of sixty mesh. Moisture was determined by drying the specimens of flour at 105°C. for 10 hours and the percentage composition of the other constituents was determined according to the method recommended by the Association of Official Agricultural Chemists. Table I gives the results of the analysis :—

TABLE I.

Pulse.	Moisture (per cent).	Total nitrogen (per cent).	Protein N $\times$ 6.25 (per cent).	Ether extra- ctives (per cent).	Ash (per cent).	Crude fibre (per cent).	Carbohy- drate ; by diff. (per cent).
<i>Phaseolus mungo</i> ..	15.0	3.72	23.26	2.88	3.39	0.81	54.66
<i>Lens esculenta</i> ..	15.14	3.62	22.60	2.09	1.78	0.86	57.53
<i>Lathyrus sativa</i> ..	12.1	5.15	52.2	2.9	2.1	0.90	49.80

#### PERCENTAGE OF TOTAL NITROGEN EXTRACTIBLE BY DIFFERENT SOLVENTS.

The flours were extracted successively with distilled water, 3 per cent NaCl solution, 70 per cent alcohol, and 2 per cent caustic-soda solution.

Ten grammes of the sieved flours were taken in small stoppered bottles. Seventy c.c. of water with a few drops of toluene were introduced and the contents shaken thoroughly in a shaking machine. Extraction with each solvent was made twice and the shaking was continued for two hours for the first extraction and for one hour for the second. The contents of the bottles were filtered by suction through filter-paper pulp and the washing was done with the solvent for four times. The filter-paper pulp with the residual flour was then introduced into the shaking bottles, shaken for the second time with the same amount of the same solvent and filtered as before. The filtrate was added to the first fraction and the total nitrogen determined by the Kjeldahl method. The same process was repeated for the residue with 3 per cent salt solution, 70 per cent alcohol, and with 0.2 per cent

caustic soda, in succession. Each determination was made in duplicate. The mean results are given in Table II :—

TABLE II.

Pulse.	PERCENTAGE OF TOTAL NITROGEN EXTRACTED WITH				
	Water.	3 per cent NaCl.	70 per cent alcohol.	0.2 per cent NaOH.	Total N <sub>2</sub> extracted (per cent).
<i>Phaseolus mungo</i> ..	18.0	50.0	1.8	22.5	92.3
<i>Lens esculenta</i> ..	25.9	44.0	1.8	20.6	92.3
<i>Lathyrus sativa</i> ..	45.2	36.2	1.2	11.0	93.6

A small percentage of nitrogen is not accounted for and probably represents nitrogenous substances unextractable with these solvents. It is evident that in the case of green gram and lentil the highest percentage of nitrogen is obtained in the salt extract, globulins forming the largest amount of the proteins of both these pulses. The alkali also extracts a fairly large amount of nitrogen. Hence the globulins and glutelins were taken as the first object of study in the case of green gram and lentil. In the case of *Lathyrus sativa* the largest percentage of nitrogen is obtained in the water and salt extracts, though the alkali extracts also contain a certain amount. Hence the albumin, globulin, and glutelin were the objects of study in this case.

The water-soluble proteins in the case of *Lathyrus sativa* are found to undergo very rapid changes on keeping the extract for a short time. The fresh solution is neutral to litmus but it gradually develops acidity and precipitation takes place. The change is so rapid that a considerable coagulation takes place within half an hour of extraction. Similar difficulty was encountered in the case of field pea by Osborne *et al.* (*loc. cit.*). In the case of green gram and lentil, water, in our preliminary experiments, was found to extract a larger percentage and salt solution a smaller percentage of nitrogen than the values in Table II. A typical result is given below :—

Pulse.	PERCENTAGE OF TOTAL NITROGEN EXTRACTED WITH	
	Water.	Salt.
Green gram ..	25	44
Lentil ..	50	18.2

On examination it was found that the aqueous extract in these experiments was, after shaking, left unfiltered for more than 24 hours and that the extract had developed acidity. It is probable that the small amount of acid formed was enough to peptize the proteins. So in later experiments the extract, after shaking, was separated from the residue as quickly as possible.

#### EFFECT OF CONCENTRATION OF SALT SOLUTION.

The powdered pulses were extracted with salt solutions of different concentrations. Five grammes of the flour were repeatedly extracted with 40 c.c. of the salt solution of a definite concentration so long as nitrogen was found in the extract.

Table III indicates the results obtained :—

TABLE III.

Concentration of salt solution (per cent) :—	1	1.5	2	2.5	3	3.5	4	4.5	5	5.5	6
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#### (A) *Phaseolus mungo*.

Per cent protein extracted.	..	60.8	70.8	74.1	74.4	74.0	73.4	73.1	72.5	72.1	70.1
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#### (B) *Lens esculenta*.

Per cent protein extracted.	..	63.3	77.9	83.3	83.0	82.5	81.2	80.1	77.6	..	..
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#### (C) *Lathyrus sativa*.

Per cent protein extracted.	79.5	..	81.4	..	76.8	..	76.5	..	71.5	..	..
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In the case of the *Phaseolus mungo* the maximum amount of protein is extractible with 3 per cent salt solution, while 2.5 per cent salt solution is the best concentration in the case of the *Lens esculenta*. In the case of *Lathyrus sativa* 2 per cent salt solution is the optimum concentration. This was borne in mind in the case of subsequent large scale extraction.

#### EXTRACTION AND PURIFICATION OF PROTEINS.

##### (1) *Aqueous extract (albumins).*

(A) *Phaseolus mungo*.—One and a half kilograms of flour were extracted with 7½ litres of distilled water for two hours and allowed to settle for about an hour when the partially clear liquid was decanted off and the process of extraction with water repeated twice. The aqueous extract was freed from insoluble residues by



centrifuging in a powerful super-centrifuge (36,000 revolutions per minute). Thus the difficulty in filtration, encountered by many, was easily avoided.

It is interesting to note that a protein was found to separate from the clear extract when it was preserved in a refrigerator. The protein coming out of the solution was not a denatured one as it was soluble in water even at 40°C. This fraction of the protein was purified by taking advantage of this effect of cooling and by dialysis for three days in a current of distilled water. The protein fraction, which separated on cooling from the centrifuged clear dialysate, was washed with 70 per cent alcohol, absolute alcohol, and ether, and was stored up as 'albumin A'.

The clear solution, after removal of albumin A, was then made half-saturated with ammonium sulphate. The precipitate that came down was suspected to be globulin and was dissolved in 4 per cent salt solution but the whole solution became black within a few hours, and isolation was not possible.

Now the half-saturated clear solution was centrifuged and saturated with ammonium sulphate. A precipitate came down which was soluble in water. This solution was then dialysed for about six days in tap water, and three days in a current of distilled water. A portion separated on dialysis. This was probably a globulin and was preserved for analysis. The clear dialysate was heated on water-bath very slowly and coagulation of the protein occurred at 58°C. Temperature was kept constant and the coagula were separated, washed with alcohol and ether, and stored as  $\alpha$  albumin. The remaining clear solution gave another albumin fraction coagulable at 75°C. It was stored as  $\beta$  albumin.

(B) *Lens esculenta*.—Though the percentage of total nitrogen extractible with water in the case of *Lens esculenta* is about one and a half times that of *Phaseolus mungo*, the yield of proteins in an aqueous extract of an equal amount of flour was too small to proceed with isolation or chemical analysis.

The nitrogen extracted by water from *Lens esculenta* appears to be non-protein nitrogen and seems to come from peptone and polypeptides.

(C) *Lathyrus sativa*.—The rapid coagulation of the water extract caused much difficulty in extraction and subsequent processes. A study of the process of extraction suggested that it was best to avoid the extraction with water and to treat the powdered meal direct with sodium chloride solution and to bring about a separation of the different proteins by fractional precipitation with ammonium sulphate.

## (2) Extraction with salt solutions (globulins).

(A) *Green gram and lentil*.—The residue from aqueous extract was treated with 3 per cent NaCl in case of green gram and 2.5 per cent salt solution in the case of the lentil, and the insoluble residue separated by the super-centrifuge.

In isolating the globulins of rice, Jones and Gersdorff (1927) used the method of precipitation by (a) making the solution 0.3 per cent saturated with ammonium sulphate and subsequent acidification with acetic acid, and by (b) fractional heat coagulation. Both the methods would make the protein denatured.

The process of alternate solution and precipitation by dilution was, therefore, adopted. The salt extract was dialysed for five days and the separated protein separated by the super-centrifuge. The protein was redissolved in the salt solution and precipitation was then effected by dilution with seven times its volume of water. The globulin that came out was dissolved again in salt solution and reprecipitated by dialysing for six days in tap water and then for four days in distilled water. The protein was washed with alcohol and ether, and preserved for analysis.

(B) *Lathyrus sativa*.—One and a half kilograms of flour were extracted with 7.5 litres of 2.5 per cent NaCl for half an hour and allowed to settle for about an hour. The partially clear liquid was then decanted off and the process of extraction with salt solution was repeated twice. The insoluble residue was removed by the super-centrifuge. The clear extract was then subjected to fractional precipitation with ammonium sulphate which yielded three distinct proteins at 3/10, 1/2, and 3/4 saturation, respectively. These fractions were separately redissolved in dilute ammonium sulphate (by adding some water to the precipitate) and each fraction was fractionated a second time. The different fractions were then dialysed for five days in tap water and then for four days in distilled water. The proteins were then washed with alcohol and ether.

### (3) *Extraction with 70 per cent alcohol (prolamins).*

(A) *Green gram and lentil*.—The residue after extraction with salt solution was boiled for two hours with 70 per cent alcohol under reflux. The insoluble matter was separated by the centrifuge and the dissolved protein was precipitated by increasing the percentage of alcohol to about 95. Purification of the protein was effected by taking advantage of its solubility in 70 per cent alcohol and its insolubility in 95 per cent alcohol. It was then washed with absolute alcohol and ether.

(B) *Lathyrus sativa*.—The alcoholic extracts were very low in nitrogen content. Moreover, the proteins, if there were any, were not precipitated by the usual methods. The *Lathyrus sativa* thus appears to contain very little prolamins. The extraction with alcohol was avoided and the residue from the salt extract was treated direct with caustic-soda solution.

### (4) *Extraction with alkali (glutelins).*

(A) *Green gram and lentil*.—The residue from (3) was extracted thrice with 0.2 per cent caustic-soda solution and clear extract was obtained by cooling it in a refrigerator and then centrifuging it. On making it 0.04 saturated with ammonium sulphate and slightly acidifying with hydrochloric acid, flocculent precipitate was obtained. Csonka and Jones (1927) isolated two glutelins from wheat flour; the first was obtained by making the alkali extract 0.018 to 0.02 saturated with ammonium sulphate and the second was obtained when the remaining solution was made 0.16 saturated with ammonium sulphate. We could, however, obtain only one glutelin. The flocculent precipitate obtained in our case was redissolved in 0.2 per cent NaOH solution and reprecipitated. The procedure was again

repeated. A small amount of prolamin coming with glutelin was removed by shaking and washing the final glutelin with 70 per cent alcohol. The protein thus extracted was washed with absolute alcohol and ether.

(B) *Lathyrus sativa*.—The residue from the salt extract was treated direct with 0.2 per cent caustic-soda solution successively for three times. By making the alkaline extract 0.03 saturated with ammonium sulphate a glutelin was precipitated and no other glutelin could be obtained. This was purified as in the previous case.

The different proteins extracted from the pulses are given in Table IV :—

TABLE IV.

Nature of the protein.	From <i>Phaseolus mungo</i> .	From <i>Lens esculenta</i> .	From <i>Lathyrus sativa</i> .
1. Water-soluble ..	(i) Albumin A—on keeping in the cold.  (ii) Albumin $\alpha$ —on full saturation with ammonium sulphate coagulable at 58°C.  (iii) Albumin $\beta$ —on full saturation with ammonium sulphate coagulable at 75°C.	Appears to consist of non-protein nitrogen.	..
2. Salt-soluble ..	(i) Globulin .. ..  (ii) Globulin separated from (ii) in 1 on dialysis.	(i) Globulin	(i) Precipitated on 3/10 saturation with ammonium sulphate.  (ii) Precipitated on 1/2 saturation with ammonium sulphate.  (iii) Precipitated on 3/4 saturation with ammonium sulphate.
3. 70 per cent alcohol-soluble.	Prolamin .. ..	Prolamin ..	Traces.
4. Alkali-soluble ..	Glutelin .. ..	Glutelin ..	Glutelin

## ELEMENTARY ANALYSIS OF PROTEIN.

The analyses of proteins were made according to the micro-method of Pregl. Table V shows the results of globulin and glutelin from *Lens esculenta* :—

TABLE V.

*Lens esculenta.*

(Calculated on ash- and moisture-free basis.)

Per cent.	GLOBULIN.			GLUTELIN.		
	(i).	(ii).	Mean.	(i).	(ii).	Mean.
Carbon     ..	51.53	51.20	51.37	50.93	50.84	50.89
Hydrogen    ..	7.92	7.74	7.83	8.27	8.15	8.21
Nitrogen    ..	15.83	16.0	15.92	13.65	13.67	13.66
Sulphur     ..	..	..	Absent	0.69	..	0.69
Ash         ..	..	..	1.38	..	..	2.42
Moisture    ..	..	..	3.0	..	..	3.32

Table VI gives the nitrogen content of the various proteins obtained by the micro-Dumas method :—

TABLE VI.

*Nitrogen content of isolated proteins by the micro-Dumas method.*

Protein.	Source.	Per cent of nitrogen (fresh basis).	Mean.	Ash (per cent).	Moisture (per cent).	Nitrogen (per cent) (ash- and moisture-free basis).
Globulin	Green gram	$\left\{ \begin{array}{l} 1 \\ 2 \end{array} \right.$ 14.35 14.35	14.35	2.15	3.83	15.86
Glutelin	"	$\left\{ \begin{array}{l} 1 \\ 2 \end{array} \right.$ 15.21 15.17	15.19	2.28	3.62	16.14
Prolamin	"	$\left\{ \begin{array}{l} 1 \\ 2 \end{array} \right.$ 12.08 12.09	12.085	1.56	3.70	12.76
Albumin A	"	$\left\{ \begin{array}{l} 1 \\ 2 \end{array} \right.$ 14.87 14.87	14.87	0.59	2.38	15.33
Albumin $\alpha$	"	$\left\{ \begin{array}{l} 1 \\ 2 \end{array} \right.$ 14.97 15.02	14.99	0.38	2.47	15.43
Albumin $\beta$	"	$\left\{ \begin{array}{l} 1 \\ 2 \end{array} \right.$ 15.44 15.26	15.35	0.62	2.91	15.91
Globulin	Lentil	$\left\{ \begin{array}{l} 1 \\ 2 \end{array} \right.$ 15.30 15.14	15.22	1.38	3.0	15.92
Glutelin	"	$\left\{ \begin{array}{l} 1 \\ 2 \end{array} \right.$ 12.86 12.88	12.87	2.42	3.32	13.65
Prolamin	"	$\left\{ \begin{array}{l} 1 \\ 2 \end{array} \right.$ 11.36 11.35	11.35	2.39	3.53	12.07

## NITROGEN DISTRIBUTION OF THE PROTEINS.

The nitrogen distribution of the proteins was determined by the van Slyke (1911) method as modified by Plimmer and Rosedale (1925) with minor modifications in detail. The results are indicated in Tables VII-A, VII-B and VII-C:—

TABLE VII-A.

*Phaseolus mungo.*

PROTEIN INVESTIGATED:—  EXPRESSED:—	GLOBULIN.		GLUTELIN.	
	in mg.	as per cent of total nitrogen.	in mg.	as per cent of total nitrogen.
Total nitrogen ..	337.54	..	349.46	..
Humin (acid insoluble) ..	3.74	1.11	8.662	2.48
Humin (acid soluble) ..	3.78	1.12	4.998	1.43
Amide .. ..	51.40	15.24	41.24	11.80
<i>Diamino fraction—</i>				
Arginine .. ..	28.77	8.53	17.04	4.88
Histidine .. ..	10.56	3.13	20.30	5.81
Cystine .. ..	2.35	0.69	4.58	1.30
Lysine .. ..	20.28	6.01	17.45	4.99
<i>Monc-amino fraction—</i>				
Amino .. ..	202.82	60.08	202.2	57.86
Non-amino ..	9.35	2.77	30.2	8.64
TOTALS ..	333.05	98.68	346.65	99.19

TABLE VII-B.

*Lens esculenta.*

<div> <div>PROTEIN INVESTIGATED:—</div> <div>EXPRESSED:—</div> </div>	GLOBULIN.		GLUTELIN.	
	in mg.	as per cent of total nitrogen.	in mg.	as per cent of total nitrogen.
Total nitrogen .. ..	409.27	..	544.29	..
Humin (acid insoluble) ..	1.62	0.397	11.79	2.17
Humin (acid soluble) ..	5.75	1.40	11.54	2.12
Amide .. ..	26.5	6.48	62.27	11.44
<i>Diamino fraction—</i>				
Arginine .. ..	51.52	12.58	50.72	9.33
Histidine .. ..	9.11	2.22	5.23	0.96
Cystine .. ..	<i>Nil</i>	<i>Nil</i>	4.56	0.84
Lysine .. ..	56.27	13.75	56.82	10.44
<i>Mono-amino fraction—</i>				
Amino .. ..	112.2	27.41	283.00	52.0
Non-amino .. ..	142.9	34.9	54.90	10.09
TOTALS .. ..	405.87	99.14	540.83	99.39

TABLE VII-C.

*Lathyrus sativa.*

Protein precipitated at saturation :—	EXPRESSED AS PERCENTAGE OF TOTAL NITROGEN.		
	3/10 ammonium sulphate.	1/2 ammonium sulphate.	3/4 ammonium sulphate.
Humin (acid insoluble) ..	1.05	0.71	0.37
Humin (acid soluble) ..	1.31	1.25	0.61
Amide .. ..	10.52	10.21	6.12
<i>Diamino fraction—</i>			
Arginine .. ..	5.74	3.93	10.45
Histidine .. ..	2.83	1.45	8.24
Cystine .. ..	0.71	0.53	0.25
Lysine .. ..	7.35	12.83	10.75
<i>Mono-amino fraction—</i>			
Amino .. ..	60.61	61.51	40.11
Non-amino .. ..	9.38	7.23	22.45
TOTALS ..	99.5	99.65	99.35

*Tyrosine and tryptophane.*

Tyrosine and tryptophane were determined from the alkali hydrolysate of 0.1 g. of the proteins according to the colorimetric method developed by Folin and



Marenzi (1929). Table VIII gives the tyrosine and tryptophane contents of the proteins:—

TABLE VIII.

Source of protein.	Name of protein.	Tyrosine (per cent).	Tryptophane (per cent).
Green gram ..	Globulin ..	2.71	0.43
Lentil .. ..	„ ..	3.23	0.17
Green gram ..	Glutelin ..	3.30	0.52
Lentil .. ..	„ ..	3.97	0.59
<i>Lathyrus sativa</i> ..	Precipitated at 3/10 saturation with ammonium sulphate.	3.67	0.65
<i>Lathyrus sativa</i> ..	Precipitated at 1/2 saturation with ammonium sulphate.	2.91	Trace.
<i>Lathyrus sativa</i> ..	Precipitated at 3/4 saturation with ammonium sulphate.	3.38	0.74

In the case of *Lathyrus sativa* proteins cystine was also estimated by the colorimetric method of Folin and Marenzi (*loc. cit.*), as modified by Remington (1930) in the original acid hydrolysate. The results are given in Table IX:—

TABLE IX.

*Cystine content in per cent of dry protein.*

Protein precipitated at saturation:—	3/10 ammonium sulphate.	1/2 ammonium sulphate.	3/4 ammonium sulphate.
Cystine .. ..	1.79	0.87	2.41

#### AUTOHYDROLYSIS OF PROTEIN IN THE PULSES, GREEN GRAM AND LENTIL.

As the percentage of nitrogen present as protein in the aqueous extract of *Lens esculenta* was very small compared with the total nitrogen present in the

extract, it was thought that autohydrolysis of the protein by the proteolytic enzymes present in the pulse might have taken place. Table X shows a comparative study of the two pulses, green gram and lentil, as regards the degree of autohydrolysis of the proteins of the aqueous extract:—

TABLE X.

*Strength of KOH —  $\frac{N}{14}$ . Temperature 40°C.*

Pulse.	HYDROLYSIS (C.C. KOH) AFTER			
	1 hour.	6 hours.	30 hours.	55 hours.
1. <i>Phaseolus mungo</i> ..	0.2	1.10	3.15	3.20
2. <i>Lens esculenta</i> ..	0.2	0.90	1.4	1.45

Water at 40°C. was added to 5 grammes of flour of each of the two pulses to make the volume 100 c.c. The mixture was shaken for two minutes in the thermostat; 5 c.c. of clear solution were taken out and titrated according to the method of Willstätter. This consists in titrating the known volume in 90 per cent alcoholic solution with a standard solution of KOH in 90 per cent alcohol with thymolphthalein as the indicator. Two or three drops of toluene were added in the reaction flask to prevent bacterial decomposition.

It is evident that autohydrolysis of proteins in *Lens esculenta* is less than that in green gram and is therefore not the cause of the presence of such a large amount of non-protein nitrogen in the former.

#### DISCUSSION.

It will be seen from the results of chemical analysis of proteins that the globulin from the lentil contains no cystine and its glutelin also contains a very small amount. The cystine content of the globulin and glutelin of green gram, though not very high, is distinctly higher than that of these proteins from the lentil. This might account for the lower biological values obtained with the lentil as compared with the green gram proteins and might also explain the loss of fur observed in long-period feeding experiments with the lentil. It has already been shown in a previous communication from these Laboratories (Basu, Nath and Ghani, 1936b) that the addition of cystine to the lentil diet restores the fur and causes growth in rats. It must also be noted that the lentil proteins, especially the glutelin, contain a smaller percentage of histidine than the green gram. The lentil globulin is also deficient in tryptophane. The arginine and lysine contents of the lentil are, however, higher than those of the green gram.

In Table XI the histidine, cystine, and lysine contents of the proteins of *Lathyrus sativa*, field pea, lentil, and *Phaseolus mungo* are compared. The data for field pea are from the results of Osborne *et al.* (*loc. cit.*).

TABLE XI.

Amino acids.	EXPRESSED AS PER CENT OF TOTAL NITROGEN.		
	Histidine.	Cystine.	Lysine.
<i>Lathyrus sativa</i> protein precipitated at—			
3/10 saturation ..	2.83	0.71	7.35
1/2 saturation ..	1.45	0.53	12.83
3/4 saturation ..	8.24	0.25	10.75
Vicilin (pea) ..	2.17	Not determined	5.40
Legumin (pea) ..	1.69	„ „	4.98
Legumelin (pea) ..	2.27	„ „	6.03
Globulin (mungo) ..	3.13	0.69	6.01
Glutelin (mungo) ..	5.81	1.30	4.99
Globulin (lentil) ..	2.22	Nil	13.75
Glutelin (lentil) ..	0.96	0.84	10.44

From the above table it is clear that the lysine and histidine contents of *Lathyrus sativa* proteins are not less than that of the two pulses which promote growth, viz., green gram and pea. Table VIII shows that *Lathyrus sativa* proteins are deficient in tryptophane. Compared with green gram and pea the cystine content of *Lathyrus sativa* is not very low and cystine deficiency is not, therefore, responsible for the loss of fur of rats on *Lathyrus sativa* diet. The cause is very probably a deficiency in tryptophane. In fact daily addition of tryptophane solution to the *Lathyrus sativa* diet has been found to restore the healthy appearance, and silkiness and smoothness of fur, though no enhancement of growth was observed. The failure of growth with *Lathyrus sativa* diet is to be ascribed rather to inadequate intake of food than to amino-acid make-up of its proteins. This inadequate intake of food is probably caused by some toxic factor present in small amounts in *Lathyrus sativa*.

## SUMMARY.

1. Green gram contains 23.26, lentil 22.60 and *Lathyrus sativa* 32.2 per cent proteins.

2. Percentage of total nitrogen extractible by different solvents is 92.3 in the case of both green gram and lentil and 93.6 in the case of *Lathyrus sativa*.
3. In the case of green-gram globulin the maximum amount of protein is extractible with 3 per cent salt solution while 2.5 per cent is the best concentration for the lentil and 2 per cent salt for *Lathyrus sativa*.
4. As many as seven different proteins have been isolated from the green gram but the number was only three in the case of the lentil and four in the case of *Lathyrus sativa*. It was not possible to obtain the water-soluble protein from lentil, the aqueous extract of which appears to contain only non-protein nitrogen.
5. The elementary composition of the proteins has been ascertained by the micro-method of Pregl and the nitrogen distribution has been determined by the method of van Slyke as modified by Plimmer and Rosedale. Tyrosine and tryptophane have been estimated colorimetrically.
6. Lentil proteins are markedly deficient in cystine. This accounts for the lower biological value and the loss of fur observed in long-term feeding experiments.
7. The lentil globulin is also deficient in tryptophane and the glutelin in histidine: but both the arginine and lysine contents are higher than those of the green gram.
8. *Lathyrus sativa* proteins are deficient in tryptophane.
9. Autohydrolysis of lentil proteins is less than that in the green gram and is, therefore, not the cause of the presence of such a large amount of non-protein nitrogen in the aqueous extract of the lentil.

## ACKNOWLEDGMENT.

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## BIOCHEMICAL INVESTIGATIONS ON DIFFERENT VARIETIES OF BENGAL RICE.

### Part IV.

#### BIOLOGICAL VALUE OF PROTEINS OF AMAN AND AUS RICE AND OF THEIR POLISHINGS.

#### BY THE BALANCE-SHEET AND GROWTH METHODS.

BY

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Of the three main groups of Bengal rice, Aman, Aus, and Boro, the Boro or the spring paddy is the least important as it occupies only 2 per cent of Bengal's rice area. About a quarter of the area in Bengal is devoted to Aus paddy which is sown from March to May and harvested from July to September. The Aman variety is sown from March to May and is harvested in November and December, and it occupies about two-thirds of the rice area in Bengal. The Aman and the Aus varieties are, therefore, the most important and have been the subject of previous investigations in these Laboratories (Basu and Sarkar, 1935*a* and *b*; Basu and Mukherjee, 1936). These investigations were carried out with pure-line strains and dealt with the chemical composition, effect of polishing and parboiling on the composition, and with the rate of enzymic digestibility of proteins and of carbohydrates. Aman varieties were, on the whole, found to be superior with respect to protein and fat content and also with respect to the rate of digestibility of proteins. Towards taka-diestase and pancreatic amylase Aus starch was more digestible, while with salivary amylase Aman starch was more readily digested.

It is important to find out the quality of the proteins of the two varieties of rice. Rice polishings constitute about 5 per cent by weight of husked rice and are richer in proteins, fats, and vitamin B<sub>1</sub> than the corresponding polished rice.

It would be useful to determine the nutritive value of the proteins of the polishings as well. Mr. K. McLean, Director of Agriculture, Bengal, suggested 'Bhasamanik' as a typical Aman variety and 'Dhairal' as a typical Aus variety of rice to be investigated.

#### CHEMICAL COMPOSITION.

The various samples were obtained from the paddy by the following method:—

A quantity of paddy was placed in an indigenous wooden mortar and pestle known technically in Bengal as '*dhenki*'. By slow up and down movements of the pestle caused by the foot the paddy was unhusked. Where polishing was necessary the husked rice thus obtained was further treated by the above method until the desired polished stage was obtained. Parboiled samples were prepared by allowing a quantity of paddy to be steeped in water overnight and then boiling with minimum quantity of water till the desired stage was obtained followed by drying in the sun and husking.

The air-dried rice was ground to a fine powder by a machine. Moisture was determined by drying the specimens of rice and rice-polishings at 105°C. for 10 hours and the percentage composition of other constituents was determined according to the method recommended by the Association of Official Agricultural Chemists. Table I gives the result of the analysis:—

TABLE I.

*Analysis of rice and rice-polishings used as sources of protein.*

	Sample.	Moisture (per cent).	Total nitrogen (per cent).	Protein = N 6.25 (per cent).	Fat (per cent).	Ash (per cent).	Crude fibre (per cent).	Carbohydrate by difference (per cent).
Sun-dried polished rice.	Aman (Bhasamanik).	11.70	1.11	7.12	1.72	0.85	0.29	78.32
	Aus (Dhairal).	11.40	1.00	6.25	1.01	0.91	0.17	80.26
Polishings of sun-dried rice.	Aman (Bhasamanik).	10.12	2.10	13.14	10.12	11.40	12.50	42.69
	Aus (Dhairal).	10.35	1.80	11.22	9.90	9.05	13.20	46.28

## I. BALANCE-SHEET METHOD.

*Experimental.*

The technique employed was the same as in the previous investigations from these Laboratories (Basu, Nath and Ghani, 1936*a* and *b*; Basu, Nath and Mukherjee, 1937) on the biological value of proteins of green gram and lentil. In contrast with the procedure of Basu, Nath and Ghani who used two adult male rats in the metabolism cage for each experiment, only one male rat weighing between 100 g. and 300 g. was placed in each cage. Rats in the different stages of growth were used to discover if age and body-weight had any effect on the biological value.

The composition of the nitrogen-free diet was the same as in the previous experiment of Basu, Nath and Ghani, only hardened arachis oil was replaced by butter fat (ghee). As in the previous experiments, vitamin B was provided in the form of an active yeast preparation. The protein diets were prepared by replacing the starch of the nitrogen-free diet by calculated amounts of rice or of rice-polishings.

In calculating the biological values metabolic nitrogen of the faeces was calculated from a graph as recommended by Schneider (1934).

The results of only those experiments were accepted in which the food intake and hence the intake of non-protein calories was sufficient.

*Experimental results.*

The results of experiments with nitrogen-free diet and also with diets containing the different proteins under investigation (at an approximately 5 per cent level) are arranged in Tables II to VI:—

TABLE II.

*Experiments with nitrogen-free diet.*

Experiment number.	Rat number.	Average body-weight (g.).	Change in body-weight (g.).	Food intake (g.).	Metabolic nitrogen (mg.).	Endogenous urine nitrogen (mg.).	Metabolic N <sub>2</sub> per g. of food eaten (mg.).
1	150	105	—0·70	7·7	15·7	22·7	2·039
2	155	146	—1·20	7·4	14·8	23·1	2·000
3	143	113	—1·12	3·6	10·6	22·1	2·944
4	176	226	—3·70	8·1	14·0	27·8	1·728
5	75	181	—2·25	4·0	8·4	36·7	2·100
6	47	278	—2·12	7·0	16·0	31·1	2·286

TABLE III.

*Biological value of proteins of sun-dried polished rice—Aman (Bhasamanik).*

Protein content—5 per cent (approx.).

Experiment number.	Rat number.	Body weight (g.).	Change in body-weight (g.).	Daily food intake (g.).	DAILY NITROGEN INTAKE (MG.).		DAILY FECAL NITROGEN (MG.).			DAILY URINARY NITROGEN (MG.).			Biological value (B. V.).	Mean B. V.
					Total.	True.	Total.	Endo.	Exo.	Total.	Endo.	True.		
1	150	102	+0.5	7.9	55.2	55.2	14.9	14.9	0	25.9	22.7	3.2	94	80
2	155	143	+0.6	8.5	59.4	53.7	23.7	18.0	5.7	32.5	23.1	9.4	82	
3	143	160	-0.2	8.2	69.8	69.8	20.0	20.0	0	39.0	22.1	15.9	77	
4	176	202	-3.7	10.0	70.4	69.2	19.3	18.1	1.2	39.8	27.8	12.0	83	
5	75	222	+2.0	11.6	98.5	96.2	26.3	24.3	2.0	66.5	36.7	29.8	69	
6	47	290	+1.0	12.8	109.1	106.0	32.5	29.2	3.3	57.8	31.1	26.7	75	



TABLE IV.  
*Biological value of proteins of sun-dried polished rice—Aus (Dhairat).*  
 Protein content—5 per cent (approx.).

Experiment number.	Rat number.	Body weight (g.).	Change in body-weight (g.).	Daily food intake (g.).	DAILY NITROGEN INTAKE (mg.).		DAILY FECAL NITROGEN (mg.).			DAILY URINARY NITROGEN (mg.).			Biological value (B. V.).	Mean B. V.
					Total.	True.	Total.	Endo.	Exo.	Total.	Endo.	True.		
1	150	104	+0.5	7.8	58.3	53.4	20.6	15.7	4.9	26.6	22.7	3.9	93	80
2	155	146	+0.7	9.7	72.1	66.8	24.1	19.4	5.3	36.9	23.1	13.8	80	
3	143	163	+0.4	8.5	70.2	70.2	20.7	20.7	0	38.0	22.1	15.9	77	
4	176	200	-1.2	8.7	64.2	61.7	19.0	16.5	2.5	42.2	27.8	14.4	76	
5	75	224	-1.2	7.1	58.8	55.1	18.6	14.9	3.7	50.3	36.7	13.6	75	

TABLE V.

(A) *Biological value of polishings of sun-dried rice—Aus (Dhairal).*  
Protein content—5 per cent (approx.).

Experiment number.	Rat number.	Body weight (g.).	Change in body-weight (g.).	Daily food intake (g.).	DAILY NITROGEN INTAKE (MG.).		DAILY FÆCAL NITROGEN (MG.).			DAILY URINARY NITROGEN (MG.).			Biological value (B. V.).	Mean B. V.
					Total.	True.	Total.	Endo.	Exo.	Total.	Endo.	True.		
1	143	160	+0.1	9.6	106.2	74.0	60.4	28.2	32.2	45.6	22.1	23.5	68	68
2	75	220	-1.8	8.2	91.2	52.0	57.2	18.0	29.2	53.4	36.7	16.7	68	
3	47	290	-2.2	12.0	133.5	81.8	75.7	24.0	51.7	57.3	31.1	26.2	68	

(B) *Biological value of polishings of sun-dried rice—Anan (Bhasamanik).*  
Protein content—5 per cent (approx.).

Experiment number.	Rat number.	Body weight (g.).	Change in body-weight (g.).	Daily food intake (g.).	DAILY NITROGEN INTAKE (MG.).		DAILY FÆCAL NITROGEN (MG.).			DAILY URINARY NITROGEN (MG.).			Biological value (B. V.).	Mean B. V.
					Total.	True.	Total.	Endo.	Exo.	Total.	Endo.	True.		
1	143	160	+0	10.9	97.1	77.1	42.0	22.0	20.0	40.6	22.1	18.5	76	69
2	75	230	-1.5	13.4	119.4	94.4	53.1	28.1	25.0	63.9	36.7	27.2	71	
3	47	294	-3.5	12.4	107.6	80.9	55.0	28.3	26.7	61.9	31.1	30.8	62	

TABLE VI.

(A) *Biological value of proteins of parboiled, polished rice—Aman (Blasamanik).*

Protein content—5 per cent (approx.).

Experiment number.	Rat number.	Body-weight (g.).	Change in body-weight (g.).	Daily food intake (g.).	DAILY NITROGEN INTAKE (mg.).		DAILY FECAL NITROGEN (mg.).			DAILY URINARY NITROGEN (mg.).			Biological value (B. V.).	Mean B. V.
					Total.	True.	Total.	Endo.	Exo.	Total.	Endo.	True.		
1	143	160	+1.5	11.7	81.1	78.7	25.8	23.4	2.4	39.8	22.1	17.7	77	80
2	75	228	+0.3	11.7	81.3	76.4	28.3	23.4	4.9	47.2	36.7	10.5	86	
3	47	286	-2.0	14.2	91.5	91.0	39.9	32.4	7.5	51.1	31.1	20.0	78	

(B) *Biological value of proteins of polishings of parboiled rice—Aman.*

Protein content—7.2 per cent (approx.).

Experiment number.	Rat number.	Body-weight (g.).	Change in body-weight (g.).	Daily food intake (g.).	DAILY NITROGEN INTAKE (mg.).		DAILY FECAL NITROGEN (mg.).			DAILY URINARY NITROGEN (mg.).			Biological value (B. V.).	Mean B. V.
					Total.	True.	Total.	Endo.	Exo.	Total.	Endo.	True.		
1	143	170	+2.0	11.6	135.3	108.9	60.5	34.1	26.4	65.3	22.1	43.2	60	58
2	75	230	-0.4	12.3	142.4	109.4	57.1	24.1	33.0	84.2	36.7	47.5	56	

*Digestibility and protein value.*

These are indicated in Table VII:—

TABLE VII.

*Digestibility of proteins and protein value.*

Experiment number.	Rat number.	Body-weight (g.).	Sample.	Food nitrogen intake (mg.).	Nitrogen digested (mg.).	Percentage digestibility.	Mean percentage digestibility.	Mean protein value (per cent).
1	143	..	Sun-dried polished rice—Aus (Dhairal).	70.2	70.2	100.0	94.7	4.73
2	75	..		58.8	55.1	93.7		
3	150	..		58.3	53.4	91.5		
4	155	..		72.1	66.8	92.6		
5	176	..		64.2	61.7	96.1		
1	143	..	Polishings of sun-dried rice—Aus.	106.2	74.0	69.6	62.6	4.77
2	75	..		91.2	52.0	57.0		
3	47	..		133.5	81.8	61.2		
1	143	..	Sun-dried polished rice—Aman (Bhasa-manik).	69.8	69.8	100.0	97.2	5.53
2	75	..		98.5	96.2	97.7		
3	47	..		109.1	106.0	97.1		
4	150	..		55.2	55.2	100.0		
5	155	..		59.4	53.7	90.4		
6	176	..		70.4	69.2	98.3		
1	143	..	Polishings of sun-dried rice—Aman.	97.1	77.1	79.4	77.8	7.06
2	75	..		119.4	94.4	79.0		
3	47	..		107.6	80.9	75.2		
1	143	..	Parboiled polished rice—Aman (Bhasa-manik).	81.1	78.7	97.0	94.4	5.47
2	75	..		81.3	76.4	94.0		
3	47	..		98.5	91.0	92.4		

### Discussion.

Results obtained with rats of different body-weights show that, in the case of rats weighing from 100 g. to 300 g., body-weight has got no appreciable effect on the biological value of a protein. Thus the observations of Mitchell (1924) are, in general, confirmed. However, a slight tendency for the biological value to decrease with increase in body-weight is to be observed in some cases.

Results indicated above make it quite clear that from the point of digestibility of proteins and their value in the maintenance of the nitrogenous integrity of the tissues the rice-polishings, though richer in protein content, are inferior to the polished rice from which they are derived. Thus the biological value of proteins of rice-polishings is 68 as compared with 80, the corresponding mean value for polished rice. The percentage digestibility (mean) of Aus and Aman polishings is only 70, while polished rice is almost completely digested. Polishings from Aus and from Aman varieties have the same biological value but the polishings from Aman appear to be more digestible than those from Aus (i.e., 77.8 as compared with 62.6). Since the polishings contain relatively higher amounts of protein, their protein value is not lower than that of corresponding rice; indeed in the case of Aman polishings the protein value is higher.

Digestibility and biological value of proteins of both Aus and Aman rice appear to be *identical*. Both Aus and Aman proteins are almost completely digested, and most of the digested proteins are utilized in replacing the nitrogenous tissue constituents disintegrated during metabolism, the biological value being 80.

Parboiling has no effect on the digestibility and the biological value of proteins of polished rice.

### II. BY THE GROWTH METHOD.

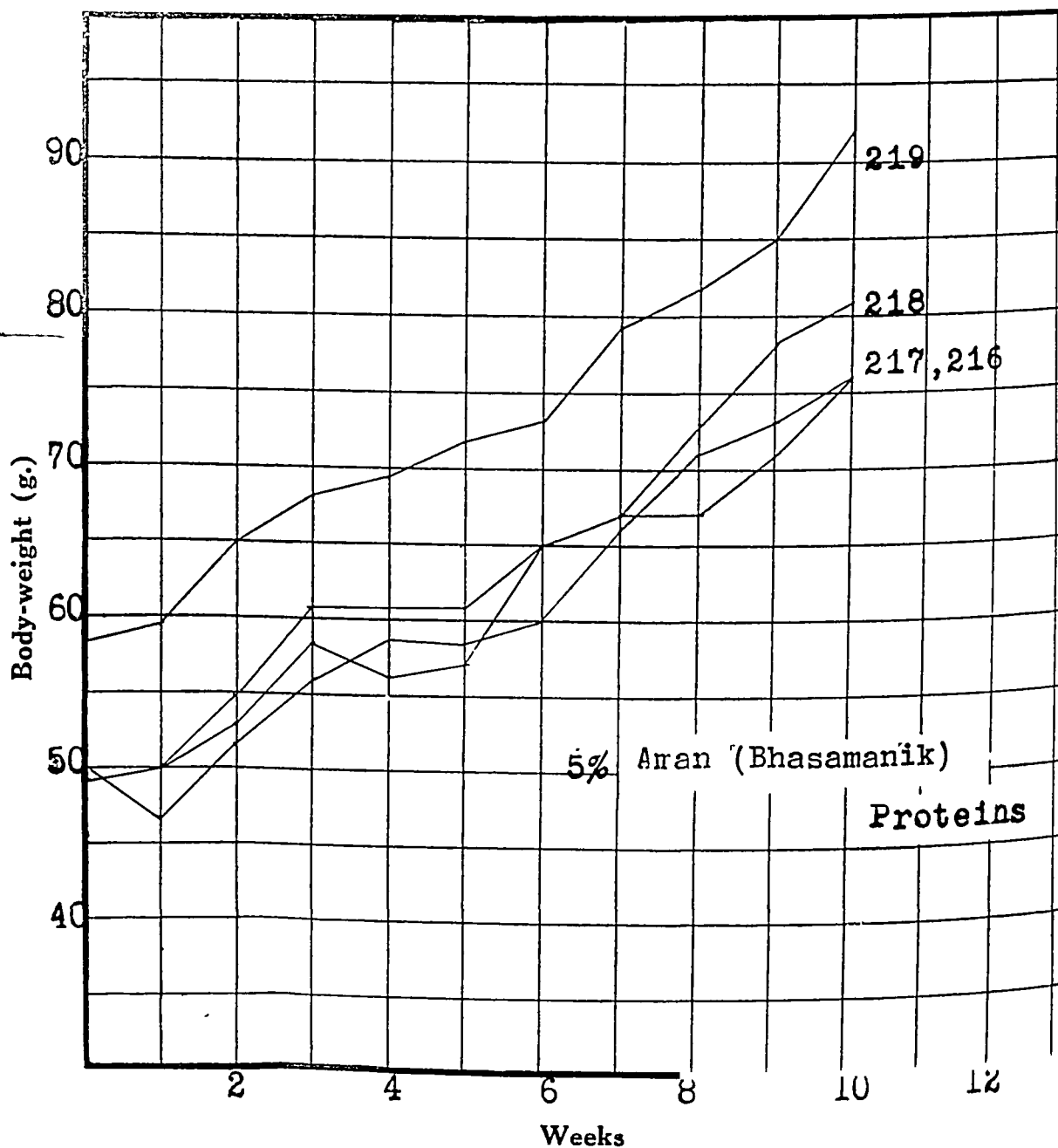
The relative values of different proteins for different functions like maintenance and growth are probably different depending on their amino-acid make-up. While the proteins of both Aus and Aman rice are of equal value for maintaining nitrogenous equilibrium during a short period, a disparity might be revealed by tests in which the growth of young rats during long periods was the criterion employed.

### Experimental.

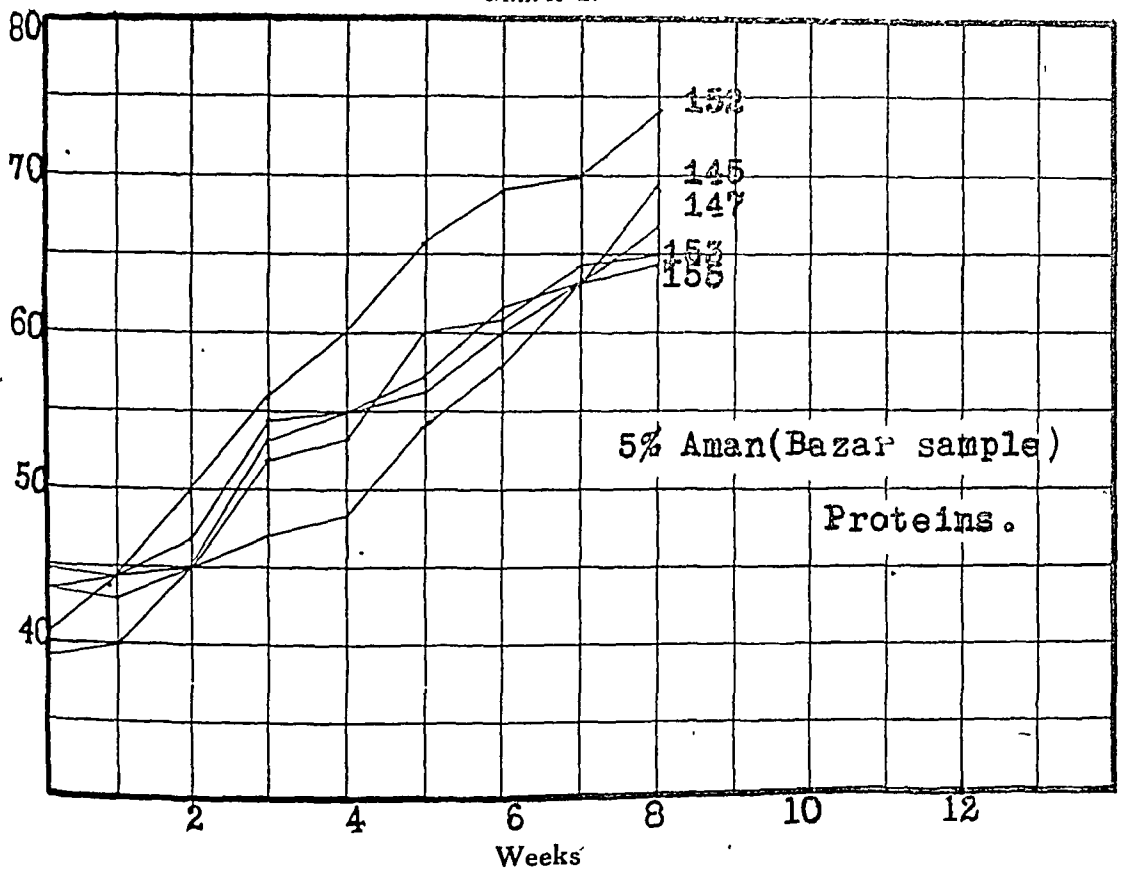
The method employed was the same as in the previous investigations from these Laboratories (Basu, Nath and Ghani, *loc. cit.*; Basu, Nath and Mukherjee, *loc. cit.*). The provision of adequate amounts of the vitamin-B complex in these experiments is essential, for otherwise the difference in growth, if any, may be caused by a difference in the quality of the protein or by a difference in the vitamin-B content. Adequate amounts of this vitamin were provided in the form of a very active yeast preparation. For details the paper by Basu, Nath and Mukherjee (*loc. cit.*) may be referred to.

The performance of the individual rats on rice and on rice-polishings diet is represented in Graphs 1 to 5 and the results are summarized in Tables VIII to XII. The composition of the diets was the same as in the balance-sheet experiments.

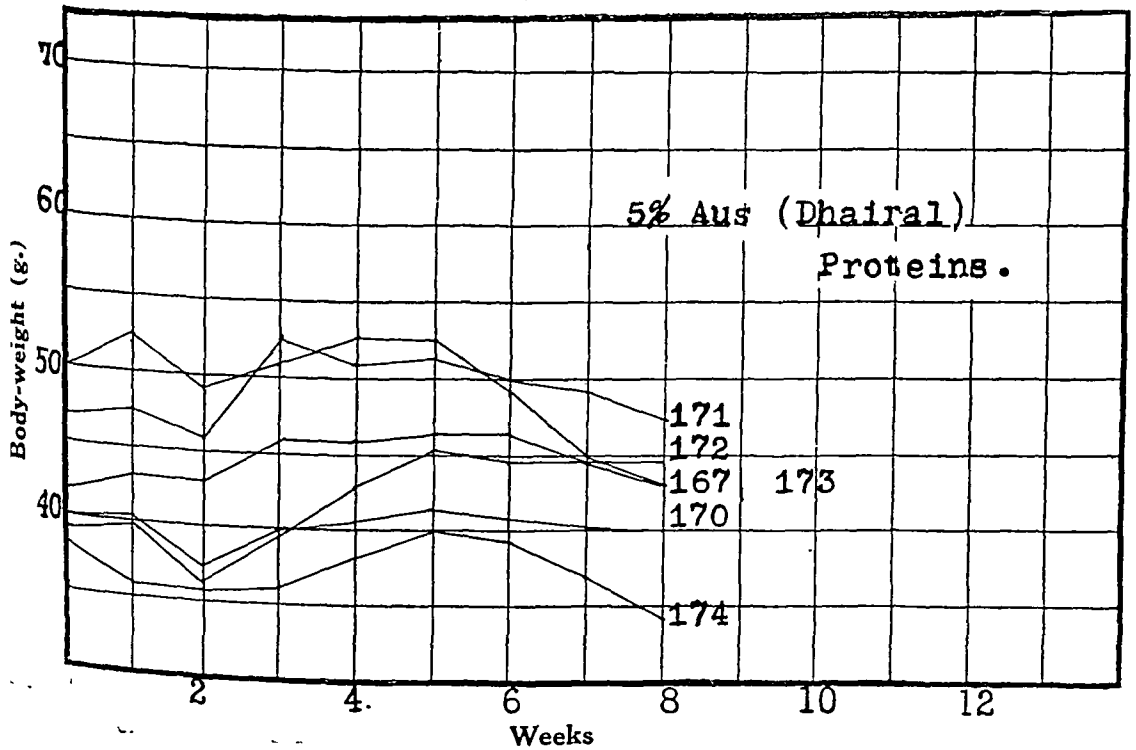
GRAPH 1.



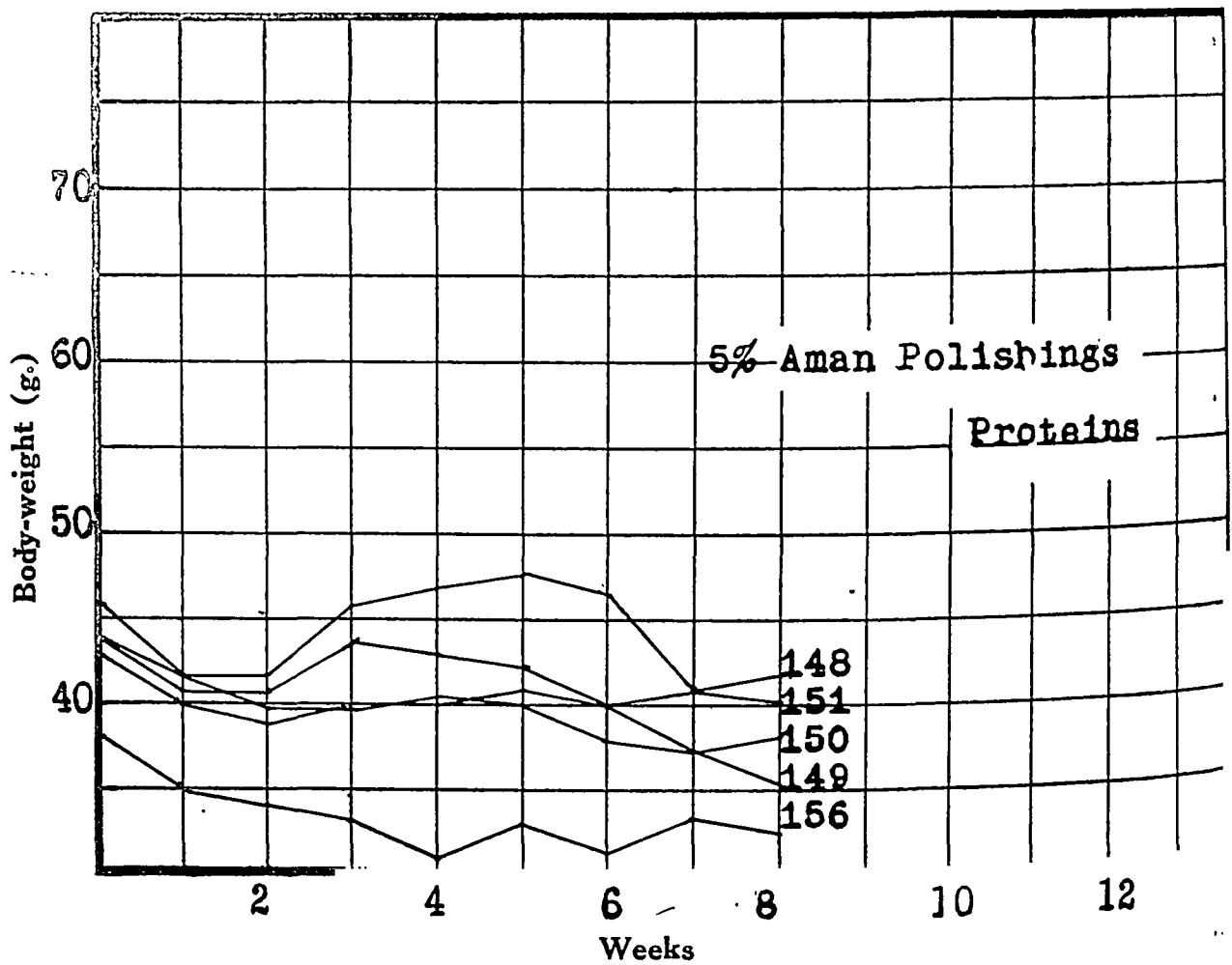
GRAPH 2.



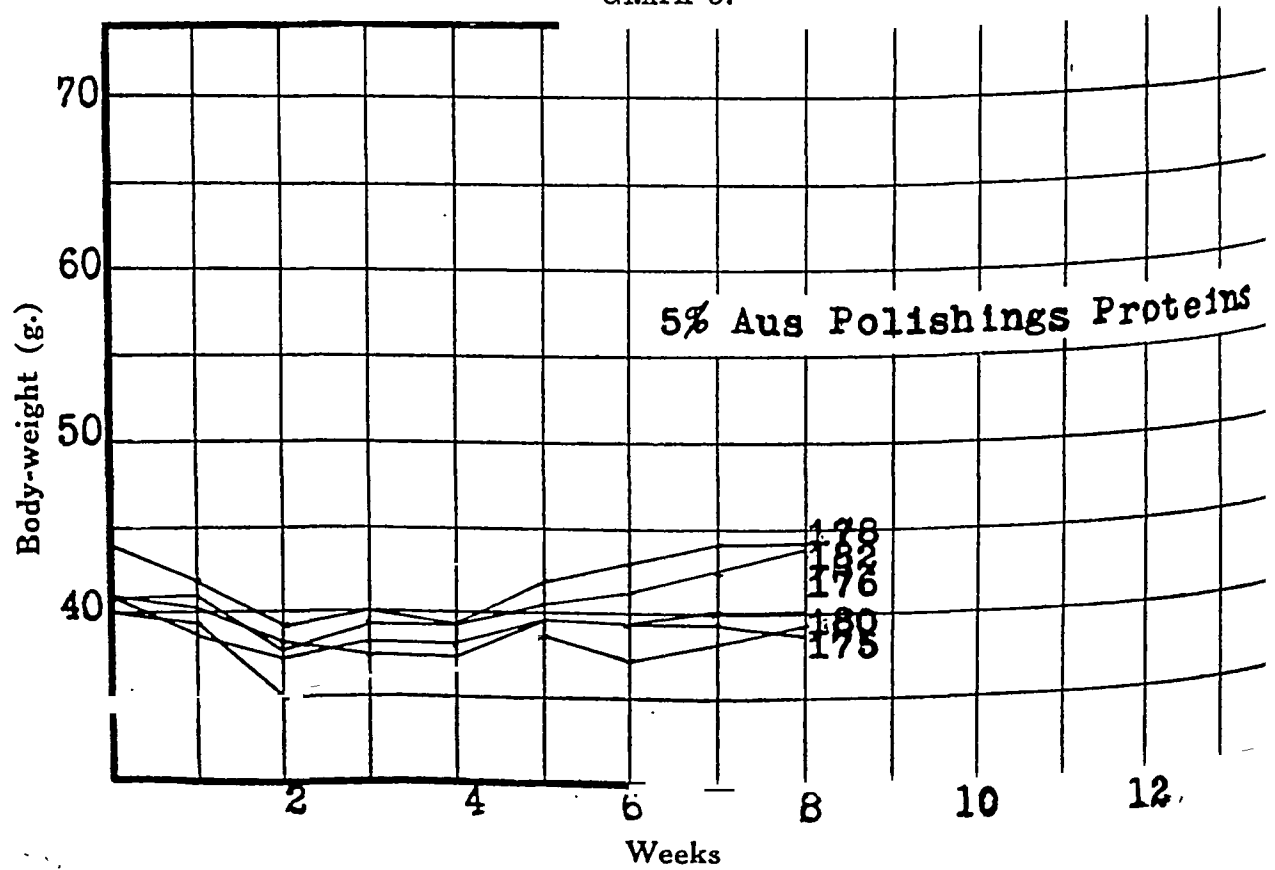
GRAPH 3.



GRAPH 4.



GRAPH 5.





*Experimental results.*

TABLE VIII.

*Experiments with proteins of Aman (Bhasamanik) rice.*

Protein content (per cent).	Rat number.	Initial weight (g.).	IN 9 WEEKS.		Gain in weight (g.).	$\frac{\text{Gain in weight}}{\text{Protein intake}} =$ B. V.	Mean B. V.
			Food intake (g.).	Protein intake (g.).			
4.4	216	50	320	11.08	26	1.84	2.01
4.4	217	47	320	14.08	29	2.06	
4.4	218	50	334	14.69	31	2.11	
4.4	219	59	368	16.19	33	2.03	

TABLE IX.

*Experiments with proteins of Aman rice (bazaar sample).*

Protein content (per cent).	Rat number.	Initial weight (g.).	IN 7 WEEKS.		Gain in weight (g.).	$\frac{\text{Gain in weight}}{\text{Protein intake}} =$ B. V.	Mean B. V.
			Food intake (g.).	Protein intake (g.).			
5	145	44	242	12.1	25	2.06	2.11
5	147	44	230	11.5	23	2.00	
5	152	44	242	12.1	30	2.48	
5	153	43	210	10.5	22	2.09	
5	155	40	247	12.3	24	1.95	

TABLE X.

*Experiments with proteins of Aus (Dhairal) rice.*

Protein content (per cent).	Rat number.	Initial weight (g.).	Food intake (g.).	Protein intake (g.).	Gain in weight (g.).	B. V.
5.2	167	52.5	125	6.50	-9.5	..
5.3	170	40.5	127	6.60	-0.5	..
5.3	171	47.5	140	7.28	0.5	..
5.3	172	39.5	135	7.02	5.5	..
5.3	173	43.5	131	6.81	-0.5	..
5.3	174	38.5	111	5.77	-4.5	..
5.3	220	51.0	146	7.59	-9.0	..
5.3	221	52.0	151	7.85	-9.0	..

TABLE XI.

*Experiments with proteins of rice-polishings—Aman (Bhasamanik).*

Protein content (per cent).	Rat number.	Initial weight (g.).	Food intake (g.).	Protein intake (g.).	Change in weight (g.).	B. V.
5.4	148	42	188	10.15	-0	..
5.4	149	40	148	7.99	-4.5	..
5.4	150	42	154	8.31	-4.0	..
5.4	151	41	190	10.26	-0.5	..
5.4	156	35	145	7.83	-2.5	..

TABLE XII.

*Experiments with proteins of rice-polishings—Aus (Dhairal).*

Protein content (per cent).	Rat number.	Initial weight (g.).	Food intake (g.).	Protein intake (g.).	Change in weight (g.).	B. V.
5.3	175	40.5	134	7.10	-2.0	..
5.3	176	38.5	149	7.89	1.5	..
5.3	178	41.0	152	8.06	3.0	..
5.3	180	39.5	139	7.36	-0	..
5.3	182	42.0	139	7.36	1.5	..

*Discussion.*

The results indicated above make it quite clear that at 5 per cent level of protein in diet, Aus rice as well as rice-polishings of both Aus and Aman varieties are unable to support any growth in young rats—a loss of weight often results although maintenance is the normal behaviour.

Proteins of Aman rice, on the other hand, cause a fairly good growth even at 5 per cent level. The value of the ratio  $\frac{\text{gain in weight (g.)}}{\text{protein ingested (g.)}} = \text{biological value}$  is as high as 2. Though the two varieties of rice, Aus and Aman, are equally efficient in maintaining nitrogen equilibrium, the Aman variety (both the pure-line strain Bhasamanik, as well as a bazaar sample) is immensely superior to Aus in promoting growth. Since adequate amounts of vitamin B and also vitamins A and D were provided in the diets, this is only to be explained by a difference in the amino-acid make-up of Aman and Aus proteins. It also leads to the assumption that amino-acid requirement for maintenance is probably different from amino-acid requirement for growth. That the amino-acid make-up of Aus and Aman proteins is different will be shown in the next paper. It is to be noted that rice-polishings which are richer in vitamin B failed to cause any growth. Osborne and Mendel (1916*a* and *b*) found that lactalbumin showed a degree of superiority over caseinogen and edestin for supporting growth, which was much greater than the superiority for maintenance. This was due to the difference in the content of lysine of which a large amount is required for growth and a relatively small amount for maintenance. In the experiments of Morgan and Kerr (1934) proteins of raw ox-muscle were found to be inferior to those of milk or cereal for maintenance whilst superior for the support of growth.

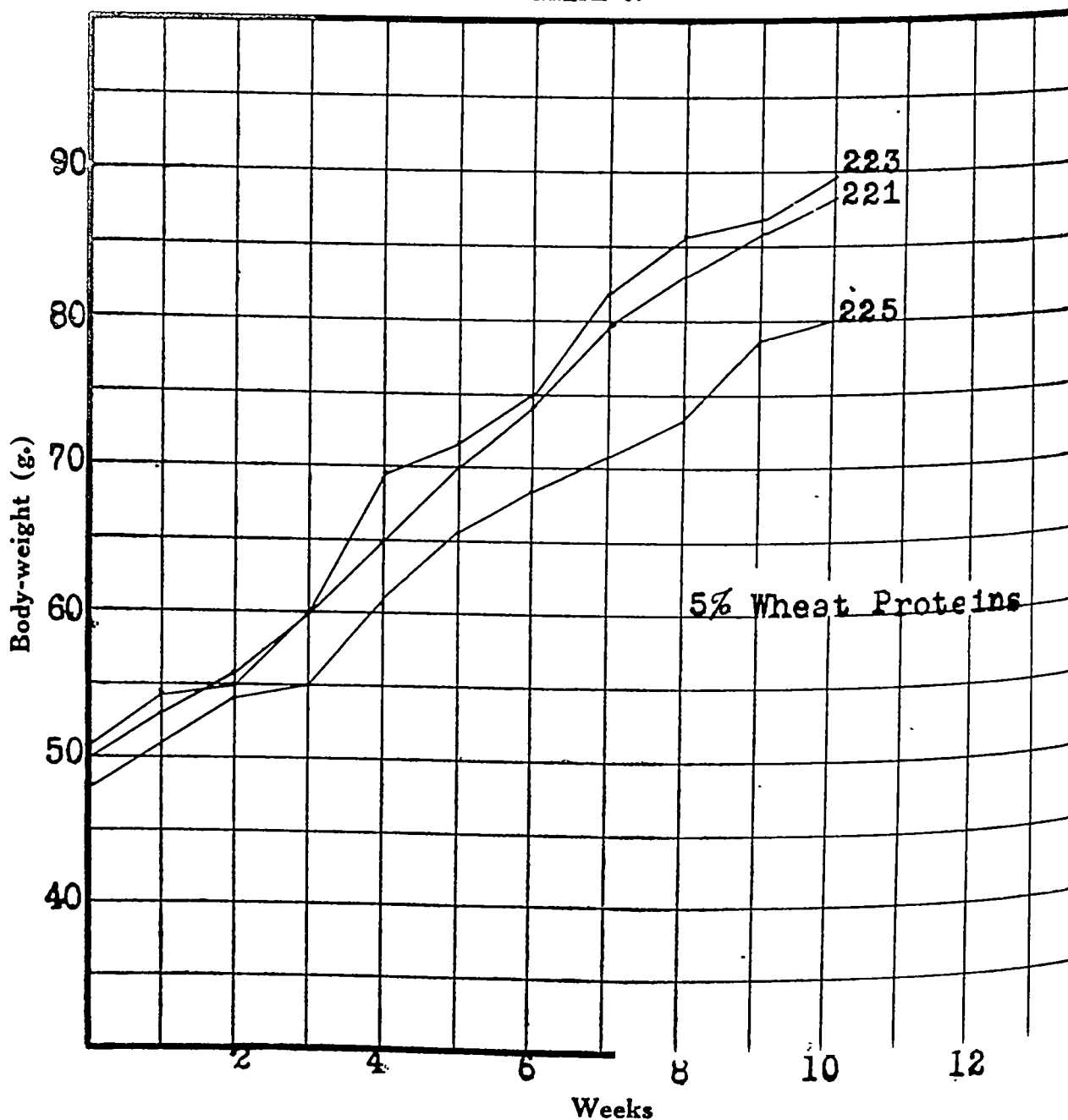
The amino-acid make-up of the Aus and Aman proteins forms the subject matter of the next investigation.

### III. COMPARATIVE VALUE OF AMAN RICE AND WHEAT PROTEINS IN PROMOTING GROWTH.

The remarkably good growth obtained with Aman rice suggested a comparison between the efficiency of proteins of Aman rice and whole wheat in promoting growth. Boas-Fixsen *et al.* (1934) have determined the biological value of wheat

protein (10 per cent protein in diet) by the method of growth and obtained the value 1.36 but no observations on the growth of young rats on a 5 per cent level of wheat protein is available. Growth experiments have, therefore, been carried out with wheat proteins (brown flour—atta) both at 5 per cent and 10 per cent levels. The performance of the individual rat is represented in Graphs 6 and 7. The results are summarized and the biological values calculated in Tables XIII and XIV:—

GRAPH 6.



GRAPH 7.

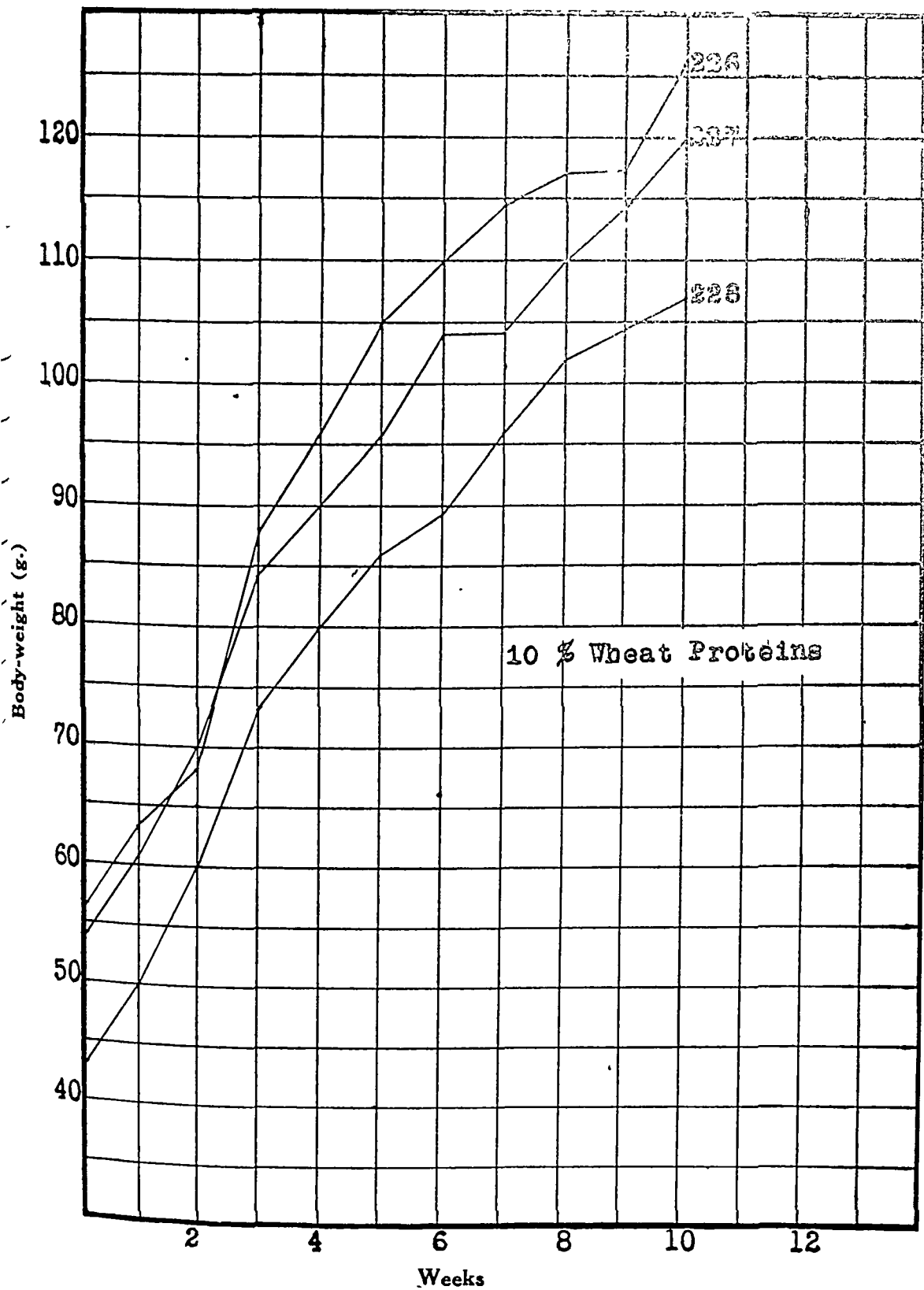


TABLE XIII.

*Experiments with wheat protein (brown flour—atta) (5 per cent).*

Protein content (per cent).	Rat number.	Initial weight (g.).	Food intake (g.).	Protein intake (g.).	Gain in weight (mg.).	Gain in weight Total protein intake B. V.	Mean B. V.
5.42	223	51	449	24.3	35	1.44	} 1.42
5.42	221	53	434	23.5	34	1.44	
5.42	225	51	383	20.7	29	1.40	

TABLE XIV.

*Experiments with wheat protein (brown flour—atta) (10 per cent).*

Protein content (per cent).	Rat number.	Initial weight (g.).	Food intake (g.).	Protein intake (g.).	Gain in weight (mg.).	Gain in weight Protein intake B. V.	Mean B. V.
9	226	63	520	46.8	63	1.34	} 1.36
9	227	61	484	43.5	59	1.35	
9	228	50	458	41.2	57	1.38	

*Discussion.*

Thus the biological value of wheat proteins at 10 per cent level is 1.36, a value identical with that of Boas-Fixsen *et al.* (*loc. cit.*), and at 5 per cent level the value is 1.42. For Aman rice the biological value at 5 per cent level is 2. For purposes of growth Aman rice proteins are, therefore, much superior to wheat proteins which are again superior to Aus rice proteins. For the maintenance of nitrogenous balance both Aus and Aman rice proteins are superior to wheat proteins, the biological value of the former at 5 per cent level being 80, while that of wheat at 5.6 per cent level is 68 (Chick *et al.*, 1935). It must not be forgotten, however, that wheat contains a much higher percentage of protein than rice.

#### IV. SUPPLEMENTARY RELATIONS BETWEEN PROTEINS OF RICE (AMAN) AND THOSE OF THE PULSES (GREEN GRAM AND LENTIL).

In practical dietetics rice and pulses are taken together and it would be interesting, therefore, to see if there is any supplementary relation between rice and pulse proteins for the maintenance of nitrogenous equilibrium and for growth. The balance-sheet method of estimating protein efficiency revealed no such supplementary relations between Aman rice and the pulses—green gram and lentil (*vide* Tables XV, XVI, and XVII). Growth method was also resorted to. Mixed diet was prepared, containing 5 per cent protein approximately, consisting of 2.5 per cent of protein from each source, i.e., rice and pulses. The performances of rats are represented in Graph 8.

The biological values are calculated in Tables XVIII and XIX.

GRAPH 8.

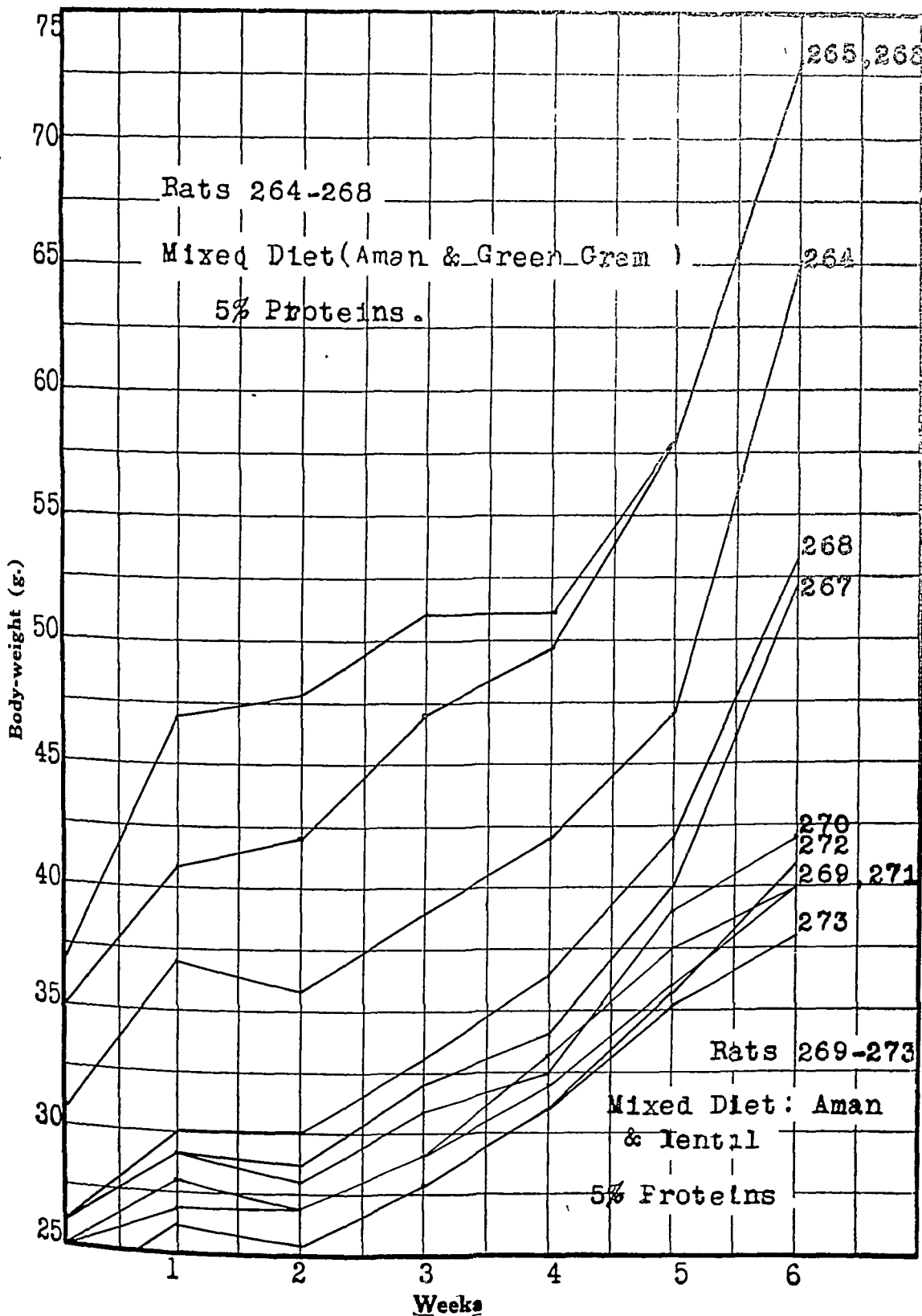


TABLE XV.  
*Biological value of mixed proteins of rice (Aman) and Phaseolus mungo.*  
Protein content—5 per cent (approx.).

Experiment number.	Rat number.	Body-weight (g.).	Change in body-weight (g.).	Daily food intake (g.).	DAILY NITROGEN INTAKE (MG.).		DAILY FÆCAL NITROGEN (MG.).			DAILY URINARY NITROGEN (MG.).			Biological value (B. V.).	Mean B. V.
					Total.	True.	Total.	Endo.	Exo.	Total.	Endo.	True.		
1	150	122	+1.5	10.0	78.6	74.2	24.9	20.4	4.5	47.8	22.7	25.1	66	64.4
2	155	164	+0.5	10.8	85.8	77.7	29.7	21.6	8.1	54.8	23.1	31.7	60	
3	143	204	+0.2	12.1	96.2	96.2	28.8	28.8	0	57.0	22.1	34.9	64	
4	176	207	+0.5	11.3	89.2	77.6	31.2	19.6	11.6	53.1	27.8	25.3	68	
5	75	248	+1.2	12.3	97.4	86.6	36.6	25.8	10.8	68.1	36.7	31.1	64	



TABLE XVI.

Biological value of mixed proteins of rice ( <i>Anan</i> ) and <i>Lens esculenta</i> . Protein content—5 per cent (approx.).													
Experiment number.	Rat number.	Body-weight (g.).	Change in body-weight (g.).	Daily food intake (g.).	DAILY NITROGEN INTAKE (mg.).			DAILY FECAL NITROGEN (mg.).			DAILY URINARY NITROGEN (mg.).		Mean Biological value (B. V.).
					Total.	True.	Total.	Endo.	Exo.	Total.	Endo.	True.	
1	150	125	0	9.6	83.0	76.2	26.3	19.5	6.8	49.6	22.7	26.9	65
2	155	166	-1.2	9.2	80.4	71.2	27.6	18.4	9.2	57.7	23.1	34.6	52
3	143	203	-2.0	8.7	75.4	70.7	30.3	25.6	4.7	56.7	22.1	34.6	52
4	176	210	-1.0	10.5	91.0	83.6	25.5	18.1	7.4	59.1	23.3	35.8	63
5	75	245	-2.2	7.8	67.7	63.2	20.9	16.4	4.5	62.3	30.7	27.6	69

Table XVII summarizes the results on supplementary relations among proteins of rice and pulses by the balance-sheet method :—

TABLE XVII.

BIOLOGICAL VALUE OF			B. V. of mixed diet. Theoretical mean.	B. V. of mixed diet. Observed mean.
<i>Phaseolus mungo</i> (5 per cent).	<i>Lens esculenta</i> (5 per cent).	Rice (Aman) (5 per cent).		
63	..	80	71.5	64.4
..	53	80	66.5	58.4

The results show that so far as maintenance of nitrogen equilibrium is concerned there is no supplementary relation whatsoever between rice and pulse proteins.

TABLE XVIII.

*Growth experiment with mixed diet of rice (Aman) and pulse (Mug).*

Rat number.	Initial weight (g.).	Food intake (g.).	Protein intake (g.).	Gain in weight (g.).	B. V.	Mean B. V.
264	37	158	7.76	28	3.60	3.26
265	41	168	8.25	32	3.87	
266	47	166	8.15	26	3.18	
267	29	159	7.81	23	2.94	
268	30	171	8.40	23	2.73	

TABLE XIX.

*Growth experiments with mixed proteins of rice (Aman) and pulse (Musari).*

Protein content (per cent).	Rat number.	Initial weight (g.).	Food intake (g.).	Protein intake (g.).	Gain in weight (g.).	Biological value.	Mean B. V.
4.91	269	27	141	6.92	13	1.87	1.76
4.91	270	29	152	7.46	13	1.74	
4.91	271	28	147	7.22	12	1.66	
4.91	272	26	151	7.41	15	2.02	
4.91	273	26	158	7.76	12	1.54	

The biological values of proteins of green gram and of lentil by the growth method have been determined in these Laboratories by Basu, Nath and Ghani (1936b). Table XX brings out the supplementary relations quite prominently:—

TABLE XX.

Biological values at about 5 per cent level of		Calculated mean B. V. of mixed diet.	Experimental mean B. V. of mixed diet.
	Rice		
Green gram (rats just maintained their weights).	2.01	1.00	3.26
Lentil (rats lost their weights).	2.01	< 1.00	1.76

The above table shows that there is a very remarkable supplementary relation between proteins of rice (Aman) and of the two pulses; moreover, the supplementary relation between rice and green-gram proteins is especially remarkable. Both Aman rice and green gram separately cause good growth: hence the supplementary relation between these two rules out the possibility of vitamin B being not provided in sufficient amounts in our experiments. Thus the practice of taking rice and pulses together, as is the custom in India, has very much to recommend it.

## SUMMARY.

(1) The biological value by the balance-sheet method of the proteins of polished rice both for Aus (Dhairal) and Aman (Bhasamanik) varieties is 80 and the digestibility is also the same (96 per cent) for the two varieties. The protein values of Aus and Aman rice are 4.73 and 5.53 respectively.

(2) The biological value of proteins of rice-polishings (both from Aus and Aman) by the balance-sheet method is 68, and the digestibility of the proteins of polishings from Aman is 77.8, while the digestibility of the proteins of Aus polishings is 62.6. The polishings from Aus and Aman have protein values of 4.77 and 7.06 respectively.

(3) Parboiling has no effect on the digestibility or the biological value of proteins of polished rice.

(4) For rats weighing from 100 g. to 300 g., their body-weight does not appear to exert any appreciable influence on the biological value of proteins.

(5) At 5 per cent level of proteins in diet, Aus rice as well as rice-polishings from Aus and Aman rice fail to cause any growth in young rats—maintenance is usually observed, although there is often a decline in weight.

(6) Aman rice at 5 per cent level of protein causes a very good growth per gramme of protein being 2.

(7) For the growth of young rats, Aman rice proteins are much superior to wheat proteins, which cause a growth of 1.42 per gramme of protein at 5 per cent level.

(8) There is a supplementary relation, so far as growth is concerned, both between rice (Aman) and green gram, and between rice (Aman) and lentil proteins—that between rice (Aman) and green gram being especially remarkable. But no supplementary relation between the Aman rice and the pulse proteins could be observed by the balance-sheet method.

#### ACKNOWLEDGMENTS.

The pure-line strains of Aman and Aus rice were very kindly supplied by Mr. K. McLean, Director of Agriculture, Bengal, to whom our best thanks are due. Thanks are also due to the Lady Tata Memorial Trust with whose help the work on the nutritive value of proteins was undertaken in these Laboratories.

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# BIOCHEMICAL INVESTIGATION OF THE PROTEINS OF VARIETIES OF PENCAB 136

## Part V.

### EXTRACTION AND CHEMICAL ANALYSIS OF THE PROTEINS OF RICE OF BOTH AMAN AND AUS VARIETIES

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IN the preceding investigation on the proteins of rice (Basu and Basak, 1937) it was shown that for the maintenance of nitrogenous equilibrium of rats, the value of proteins of both Aman and Aus varieties of rice was identical, the biological value being 80 in both cases, while for the growth of the young rats Aman was far superior to Aus. Thus the biological value of Aman rice, measured by the growth method, was 2.01, while the corresponding value for Aus rice was zero, i.e., rats just maintained their body-weights on Aus diet. Since experiments on both the varieties of rice were conducted under identical conditions, i.e., diets were similar in composition in every respect, from viewpoints of vitamins (sufficient amounts of vitamin-B complex, A and D being provided) and mineral matters, etc., except proteins, it was thought that this discrepancy between the two varieties of rice might have been due to the amino-acid make-up of the proteins in Aman and Aus rice. Aman might contain one or more 'growth essential factors' deficient or lacking in Aus. The present investigation was, therefore, undertaken to determine the amino-acid make-up of the proteins of Aman and Aus rice.

Compared with the proteins of other cereals, those of rice have been but little studied, notwithstanding the fact that rice constitutes one of the most important sources of food for a large part of the world's population. One reason for this is

doubtless due to the unusual distribution of the proteins found in rice. Unlike the proteins of other cereals, nearly all of the proteins of rice endosperm are insoluble in water, salt solution, and alcohol, and belong to the rather ill-defined class of proteins called glutelins. Consequently, most of the data recorded in the literature on rice proteins have been on a mixture of the total alkali-extractible proteins.

Rosenheim and Kajiura (1907-08) in a preliminary communication announced that rice contained small amounts of albumin (0.04 per cent) and of globulin (0.14 per cent) which coagulated respectively at 85°C. and 70°C., and relatively large amounts of a protein having the properties of glutelin to which they gave the name oryzenin.

Osborne, van Slyke, Leavenworth and Vinograd (1915) extracted polished rice with 0.2 per cent sodium hydroxide solution, and precipitated the proteins with dilute acetic acid. This protein was analysed by the van Slyke method.

Later, Hoffman (1925) isolated from 7.5 kilos of polished rice 7.5 g. of an alcohol-soluble protein which he analysed by the van Slyke method.

Tillmans and Alt (1925) state that rice flour contains neither an alcohol-soluble nor a water-soluble protein and only a small quantity of a salt-soluble protein.

Kondo and Hayashi (1926) in a series of articles reported a study of rice glutelin.

Tadokoro, Ito and Watanabe (1926) analysed the total globulins and glutelins of Japanese rice.

Larmour (1927) analysed the rice glutelin (oryzenin) by the van Slyke method.

Jones and Gersdorff (1927) have reported the results of an extensive study on globulins and glutelins of rice. Their work may be summarized as follows:—

By dialysing saline extracts they have been able to isolate a protein fraction consisting of two globulins, coagulating at 74°C. and 90°C. respectively. The concentrations of ammonium sulphate which precipitate these globulins are so close to one another that the globulins cannot be separated by this method. They are both precipitated from a 5 per cent sodium chloride solution at about 0.3 saturation with ammonium sulphate. The wide difference, however, between the temperatures at which these proteins coagulate enabled Jones and Gersdorff to separate them by fractional heat coagulation. Careful examinations of the dialysate from which the globulins had been removed failed to reveal the presence of any albumin. The authors thus agree with Tillmans and Alt (*loc. cit.*) who were likewise unable to detect the presence of an albumin. They have criticized the results of Rosenheim and Kajiura (*loc. cit.*) wherein they claim to have isolated a globulin coagulating at 70°C. and albumin coagulating at 85°C. as very unusual and difficult to explain, as rarely, if ever, an albumin has such a high coagulation temperature. They say that these two coagulation temperatures agree so closely with those found for the globulins which they isolated as to indicate that they (Rosenheim and Kajiura, *loc. cit.*) were dealing with two globulins rather than with a globulin and an albumin. Apart from the difference in their coagulation temperatures, analysis of the globulins (by these authors) revealed marked differences between them in elementary composition and in the distribution of nitrogen. The method previously used by Jones and Csonka (1927) whereby they had shown that wheat endosperm contained two different

glutelins was applied to the proteins of polished rice. The glutelin was extracted from rice powder by a 0.2 per cent sodium hydroxide solution and precipitated from this solution by making the latter 0.03 saturated with ammonium sulphate. In contrast with the wheat endosperm, they found that polished rice contained only one glutelin.

The investigations outlined above represent, as far as we are aware, about all the work that has been done on the nature and character of the different rice proteins.

The aim of the present work is to make a comparative study of the amino-acid make-up of the proteins of Aman and Aus rice. Since globulin and glutelin form about 85 per cent of the total proteins in both the varieties of rice (Table I), the present paper is concerned exclusively with the study of globulin and glutelin fractions.

#### PERCENTAGE OF TOTAL NITROGEN EXTRACTIBLE BY DIFFERENT SOLVENTS.

For the extraction of proteins, polished rice [Aman (Bhasamanik), Aus (Dhairal)] was ground to a flour sieved through sixty mesh. The composition of flour under investigation is given in Table I of the preceding paper.

Jones and Gersdorff (*loc. cit.*) have reported that a 5 per cent sodium chloride solution was the most efficient concentration for the extraction of globulin, and 0.4 per cent aqueous sodium hydroxide was the best concentration for extraction of glutelin. The flours were, therefore, extracted successively with distilled water, 5 per cent sodium chloride solution, 75 per cent cold alcohol, and 0.4 per cent caustic soda solution. Table I represents the percentages of proteins extracted by different solvents.

TABLE I.

*Percentage of protein extracted by*

Sample.	Distilled water.	5 per cent NaCl.	75 per cent cold alcohol.	0.4 per cent NaOH.	Total extraction.
Aman ..	5.8	22.6	3.7	62.7	94.8
Aus ..	7.5	29.2	3.0	55.7	95.4

It may be pointed out here that extraction with boiling aqueous alcoholic alkali (60 per cent alcohol containing 0.4 per cent sodium hydroxide) as recommended by Jones and Gersdorff could not be carried out in our case, since during the above treatment the flours swelled and coagulated into a thick pasty mass making further treatment almost improbable. The meaning of the above suggestion of Jones's is not clear.

#### EXTRACTION AND PURIFICATION OF PROTEINS.

Since a very small percentage of the total proteins could be extracted with water, this was neither suitable nor considered important for further study.

### *Globulin.*

Quantities of rice flour were extracted by shaking thrice for two hours with five to six times the volume of 5 per cent sodium chloride solution; after each extraction the whole was allowed to stand and the supernatant solution was decanted off. The combined extracts were freed from suspended impurities by centrifuging in a powerful supercentrifuge (36,000 revolutions per minute).

The globulin was precipitated from the solution by half-saturation with ammonium sulphate. The globulin was then separated from this mixture by centrifuging, washed with water, and dissolved again in sodium chloride solution, and then dialysed for about 10 days in tap water and then for three days in a current of distilled water. The precipitated globulin was then washed successively with alcohol and ether, dried and preserved for analysis.

It may be pointed out that in Aus rice only one globulin was detected coagulating at about 74°C., as found by Jones and Gersdorff for one of their rice globulins, but a second globulin coagulating at 90°C. could not be detected. In Aman rice, only one globulin was isolated coagulating at about 90°C., but no globulin coagulating at 74°C. could be detected. The present investigation is concerned with only pure-line strains supplied by Mr. McLean, Director of Agriculture, Bengal, whilst Jones and Gersdorff worked with Blue Rose variety but do not mention if they worked with pure-line strains.

### *Glutelin.*

The residue after the above treatment was shaken with five to six times the volume of 0.2 per cent sodium hydroxide solution for two hours. After allowing the mixture to stand, the supernatant liquid was syphoned and the process of extraction was repeated several times. The suspended impurities were separated by centrifuging in a supercentrifuge and glutelin precipitated by making the solution slightly acidic with acetic acid. The precipitate was separated, dissolved in 0.2 per cent alkali and again precipitated by acetic acid. This process was repeated several times ultimately dialysing in a current of distilled water for a few days. It was then washed with alcohol and ether, dried, and preserved for analysis.

### *Analysis of proteins by the van Slyke method.*

The proteins were then analysed by the van Slyke method (1911, 1915) as modified by Plimmer *et al.* (1925, 1927). Tyrosine and tryptophane were estimated by the colorimetric method of Folin *et al.* (1927, 1929).

### *Arginine.*

Van Slyke (1911) in his original paper reported estimation of arginine by boiling with 40 per cent alkali for six hours. Plimmer (1916) recommended the use of 20 per cent alkali.

The previous workers estimated arginine only in the di-amino fraction but Plimmer and Rosedale (1925) have shown that a certain amount of arginine is always to be found in the mono-amino fraction. In our investigation, therefore,



arginine was estimated in the original filtrate, after removal of ammonia but before precipitation of the phosphotungstates, by boiling with 20 per cent alkali for six hours according to the suggestion of Plimmer and Rosedale (*loc. cit.*). In order to avoid error in the calculation of the amounts of histidine and cystine, arginine was also estimated in the di-amino fraction.

### Cystine.

Van Slyke (*loc. cit.*) estimated cystine in the di-amino fraction by previous separation of phosphotungstic acid and then oxidation of the sulphur in cystine by Denis's modification of Benedict's method.

Plimmer and Lowndes (1927) have developed a method for the estimation of cystine sulphur in presence of phosphotungstic acid by the Benedict-Denis method, but they found that only 40 per cent could be estimated in the mono-amino fraction.

In this investigation, therefore, for estimation of total cystine, sulphur was determined in the original filtrate before precipitation of the phosphotungstates by the Benedict-Denis method. In order that lysine estimation might be correctly made sulphur was estimated also in the di-amino fraction.

### EXPERIMENTAL RESULTS.

The nitrogen distributions of globulins and glutelins of Aman and Aus rice are shown in Tables II and III respectively :—

TABLE II.

*Van Slyke analysis of rice globulins.*

Nitrogen.	AS PERCENTAGES OF TOTAL NITROGEN.	
	Aman (Bhasamanik).	Aus (Dhairal).
Amide .. ..	2.66	3.77
Humin (insoluble) ..	1.81	1.74
Humin (soluble) ..	1.60	1.31
Cystine (corresponding to total sulphur).	12.65	6.17
Arginine .. ..	24.56	28.00
Histidine .. ..	2.22	2.04
Lysine .. ..	4.42	4.15
Amino-N of filtrate ..	47.74	49.94
Non-amino-N of filtrate ..	2.16	2.53
TOTALS ..	99.82	99.65

TABLE III.

*Van Slyke analysis of rice glutelin.*

Nitrogen.	AS PERCENTAGES OF TOTAL NITROGEN.	
	Aman (Bhasamanik).	Aus (Dhairal).
Amide .. ..	11.42	11.17
Humin (insoluble) ..	1.21	2.18
Humin (soluble) ..	1.90	1.40
Cystine (corresponding to total sulphur).	2.00	1.66
Arginine .. ..	15.36	10.20
Histidine .. ..	3.87	3.82
Lysine .. ..	5.42	5.03
Amino-N of the filtrate ..	55.23	60.38
Non-amino-N of the filtrate	3.32	3.65
TOTALS ..	99.73	99.49

Tyrosine and tryptophane contents of the proteins were estimated colorimetrically according to Folin *et al.* (1927, 1929).

Table IV shows the amounts of tyrosine and tryptophane present in globulins and glutelins of Aman and Aus rice :—

TABLE IV.

Variety.	Protein (sample).	Tyrosine nitrogen (per cent).	Tryptophane nitrogen (per cent).
Aman ..	Globulin	2.22	0.80
Aus ..	„	2.77	0.95
Aman ..	Glutelin	3.05	0.90
Aus ..	„	2.91	1.01

## DISCUSSION.

The analyses given above would indicate that the composition of the Aus and Aman rice proteins show general similarities. Only the globulin of Aus contains much less percentage of sulphur-containing amino acids (6.17) than the corresponding Aman (12.65). Aman glutelin contains more arginine (15.36 per cent) than the Aus glutelin (10.2 per cent), whereas Aman globulin (24.56) contains less arginine than Aus globulin (28.00 per cent). But since the percentage of glutelin in rice is double the percentage of globulin, on the whole Aman rice proteins contain more

arginine than the proteins in Aus rice. Ackroyd and Hopkins (1916) reported that arginine is essential for growth and maintenance, but recent work of Rose *et al.* (1924, 1925) has definitely shown that arginine is not essential for growth and can be synthesized in the body. The difference between Aus and Aman rice proteins in causing growth is, therefore, to be sought in the difference in cystine or methionine content of the two rice proteins.

Experiments were therefore undertaken to find out if the addition of cystine or methionine to Aus rice diet in amounts corresponding to its deficiency in sulphur containing compounds as compared with Aman rice produces growth in young rats.

### *Experimental procedure.*

Rats were kept for a preliminary period of three weeks on 5 per cent Aus rice diet till the rats just maintained weight when calculated amount of amino-acid solution in question (cystine or methionine) was added to the food mixture containing Aus rice.

### RESULTS.

The results are shown in Table V and represented in Graphs 1 and 2 :—

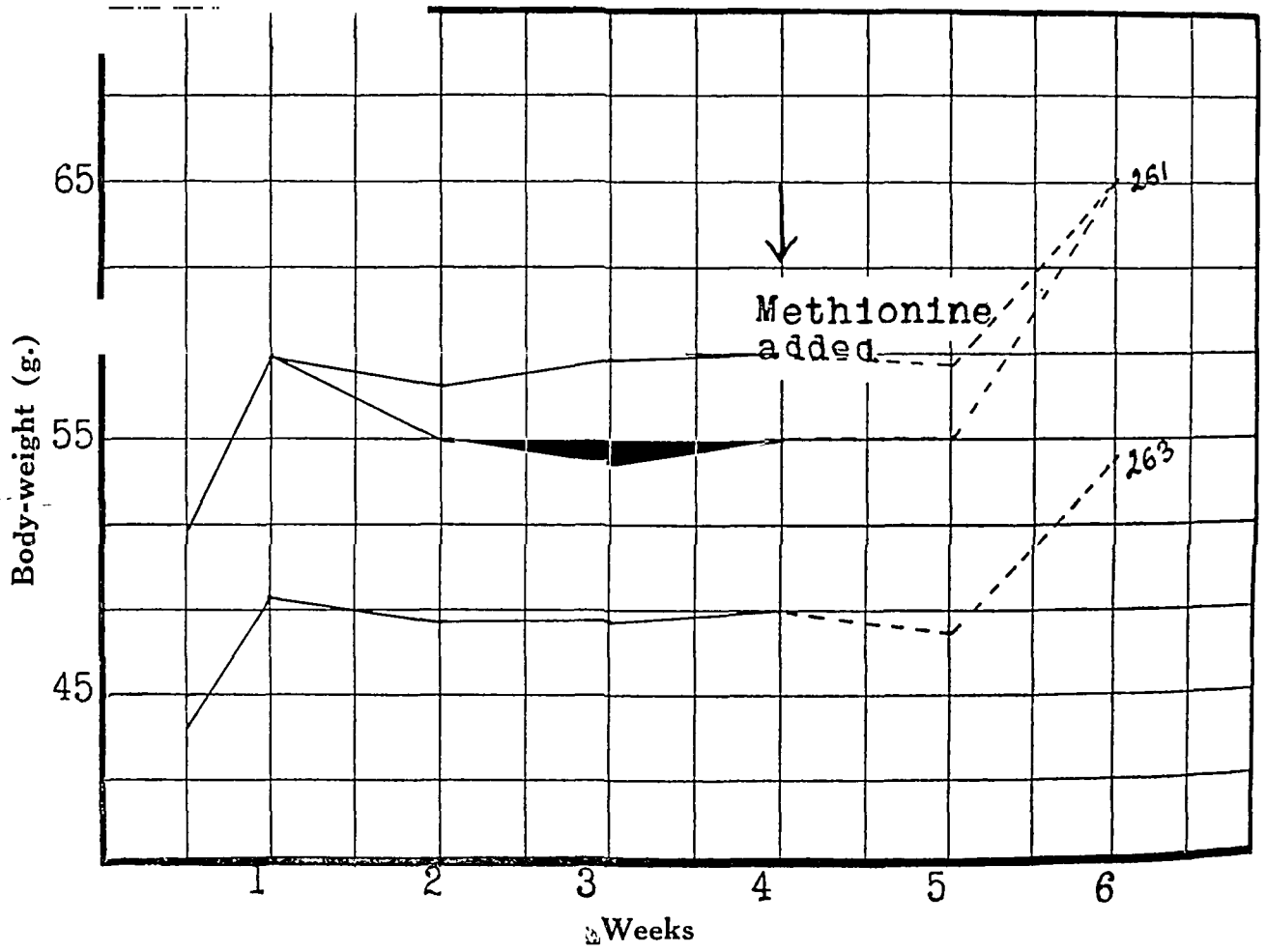
TABLE V.

*Experiment with rice (Aus) protein supplemented by amino acids.*

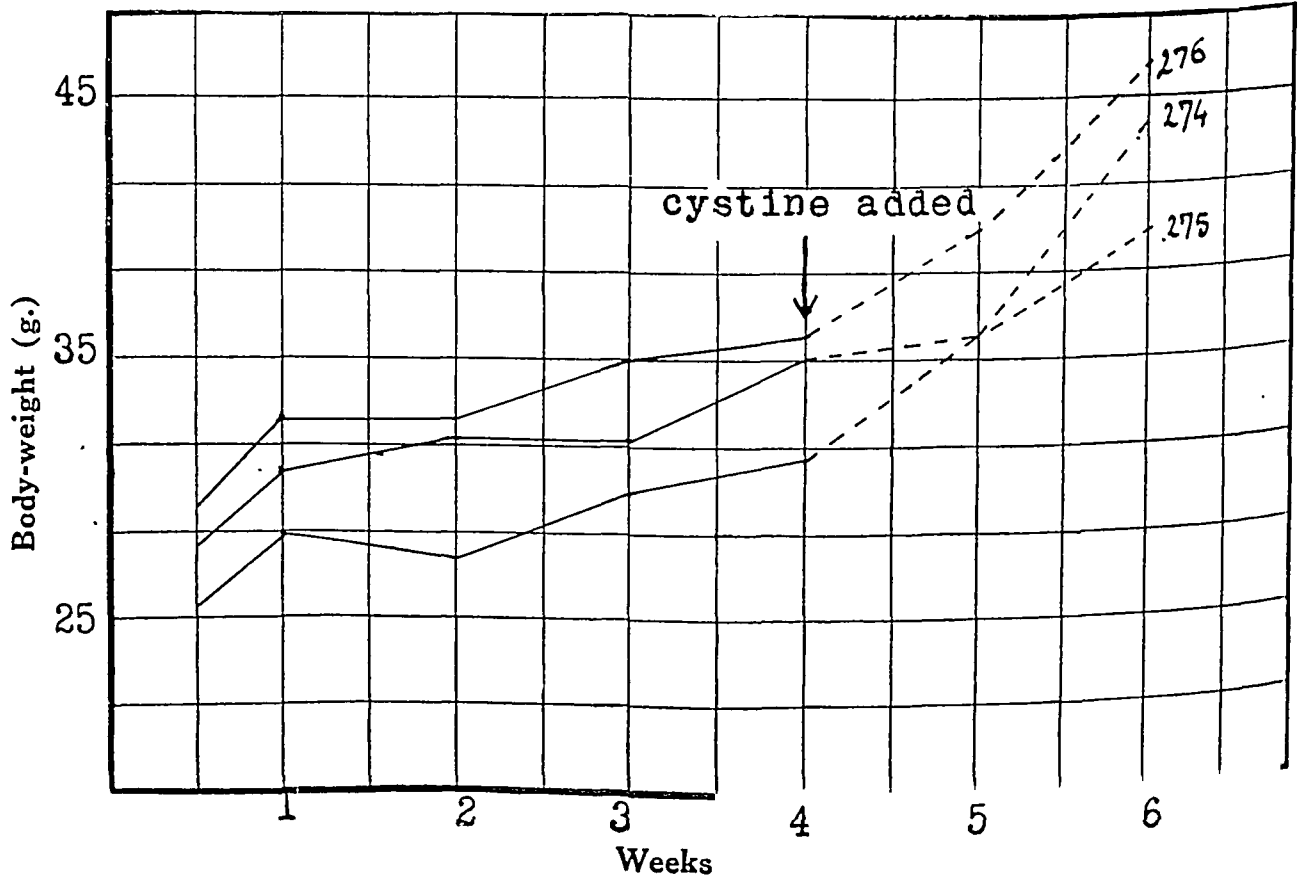
Rat number.	AUS RICE DIET (5 PER CENT PROTEIN).				METHIONINE ADDED.		
	Initial week.	1st week.	2nd week.	3rd week.	1st week.	2nd week.	3rd week.
261	45	58	55	54	55	55	65
262	45	58	57	58	58.5	58	65
263	39	49	48	48	48.5	47.5	54

Rat number.	AUS RICE DIET (5 PER CENT PROTEIN).				CYSTINE ADDED.		
	Initial week.	1st week.	2nd week.	3rd week.	1st week.	2nd week.	3rd week.
274	25	31	32	32	35	36	44
275	23	28	27	30	31	36	40
276	26	33	33	35	36	40	46

GRAPH 1.



GRAPH 2.



It will be seen from the above graphs and the table that the addition of l-cystine and d-l-methionine to Aus rice diet causes growth in young rats. Thus it is established that the difference in behaviour between Aus and Aman rice in promoting growth is to be ascribed to the sulphur-containing amino acids like cystine and methionine being not present in Aus rice in amounts sufficient to cause growth. It should be noted that Aus rice contains sufficient cystine or methionine, or both, for the maintenance of nitrogenous equilibrium. The results obtained suggest that the sulphur-containing amino-acid requirement is higher for growth than for maintenance.

#### SUMMARY.

(1) From Aman rice (Bhasamanik), water extracts 5.8 per cent, salt, 22.6 per cent, 75 per cent alcohol 3.7 per cent, and alkali 62.7 per cent; the corresponding values for Aus rice are 7.5 per cent, 29.2 per cent, 3.0 per cent, and 55.7 per cent respectively.

(2) Aus globulin coagulates at 74°C. and Aman globulin at 90°C. Aus and Aman rice globulins and glutelins have been extracted and analysed by the van Slyke method. Tyrosine and tryptophane contents of these proteins were determined by colorimetric methods.

(3) Aman rice contains more sulphur-containing amino acid and more arginine than Aus rice.

(4) Addition of either cystine or methionine in equivalent amounts causes growth in rats on Aus rice diet (protein content 5 per cent), which in its absence is incapable of promoting any growth. It is suggested that the sulphur-containing amino-acid requirement for growth is higher than that for maintenance.

#### ACKNOWLEDGMENTS.

Thanks are due to Mr. K. McLean, Director of Agriculture, Bengal, for the pure-line strains of Aus and Aman rice, and to the Lady Tata Memorial Trust with whose help the work on the analysis of proteins was undertaken in these Laboratories.

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# STUDIES IN CARBOHYDRATE METABOLISM.

## Part II.

### EFFECT OF A HIGH CARBOHYDRATE DIET CONTAINING SUGAR ON THE GLUCOSE-TOLERANCE CURVE IN THE ALBINO RAT.

BY

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AND

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In an attempt to produce a condition resembling diabetes mellitus in rats by dietary means, a group of 12 rats was fed for a prolonged period on the following diet:—

				Parts.
Raw polished rice	..	..	..	50
White flour	..	..	..	10
Sugar	..	..	..	12
Mustard oil	..	..	..	8
Ghee	..	..	..	3
Milk..	..	..	..	3
Grams, including dhal	..	..	..	5
Fish..	..	..	..	5
Root vegetables	..	..	..	2
Leafy vegetables	..	..	..	2
Condiments: extra in small quantities.				

This diet was considered to resemble in composition that consumed by the more prosperous classes in Bengal and elsewhere in India, who appear to be particularly prone to contract diabetes mellitus in middle life. Its salient

characteristics are a high content of carbohydrate, with excess of sugar and a relative deficiency of animal protein and certain vitamins. In order to discover, as the experiment progressed, whether any condition resembling diabetes was being produced in animals, glucose-tolerance tests were carried out, it being presumed that the earliest evidence of diabetes would be a decrease in sugar tolerance.

The animals on this diet did not thrive and a considerable mortality occurred. After 11 months the fasting blood sugar of 10 surviving animals was determined by an electrometric method described in a previous paper (Sankaran and Rajagopal, 1936). The results of these tests are presented in Table I:—

TABLE I.

Rat number.	Blood sugar (mg. per cent).
1	116
2	79
3	78
4	88
5	88
6	80
7	81
8	88
9	89
10	96

The average fasting blood-sugar level of a series of normal healthy rats fed on the Coonoor stock diet was found to be 101.8 mg. per 100 c.c. of blood. Hrubetz (1936) finds the blood-sugar values of 'control' rats to be 102 mg. to 108 mg. per cent.

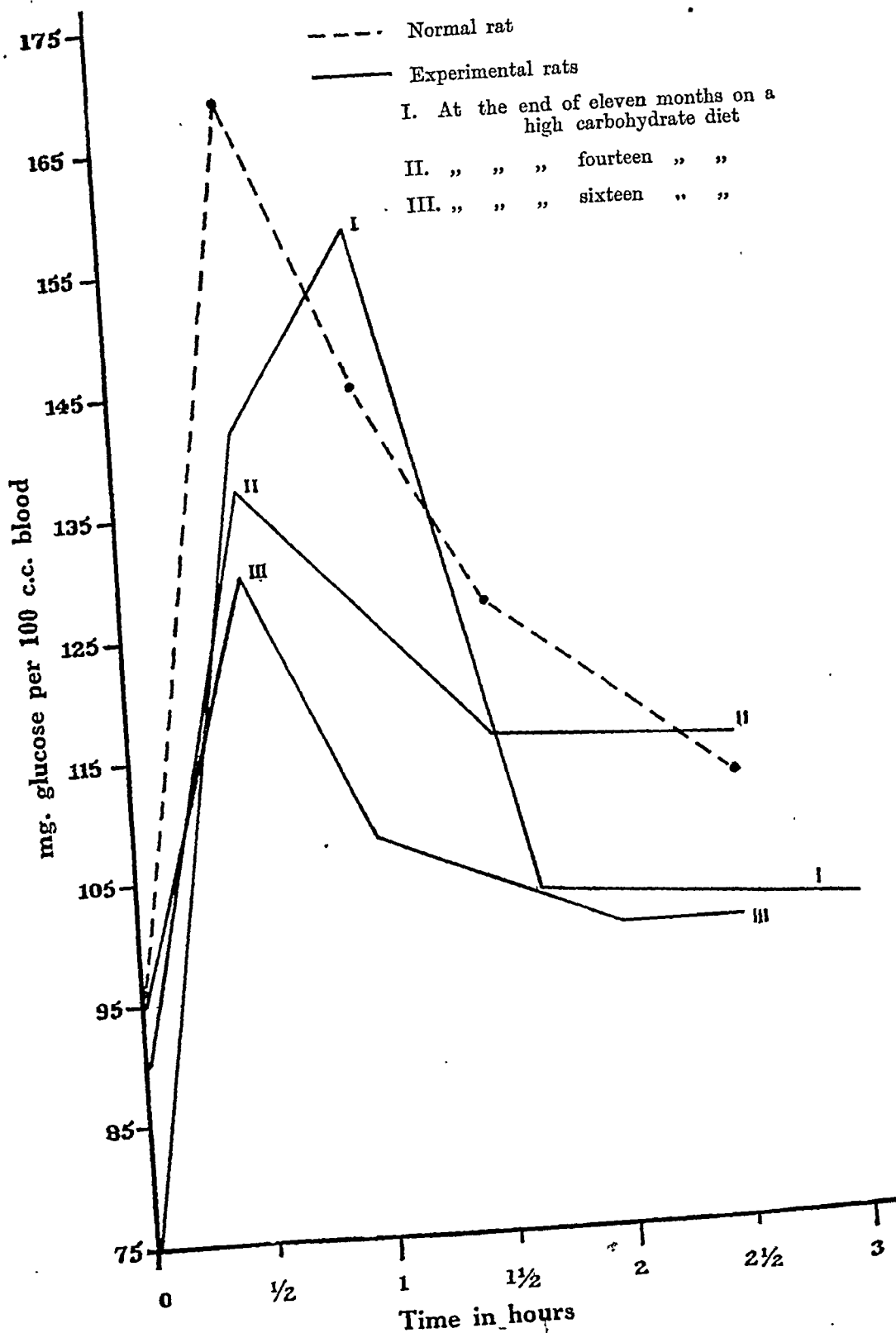
It was, therefore, evident that there was no rise in blood sugar in the experimental animals; values were, in fact, somewhat below 'normal'. It was, however, possible that the animals, while showing low blood-sugar levels, had decreased sugar tolerance. To throw light on this point, sugar-tolerance tests were carried out in accordance with the method previously described (Sankaran and Rajagopal, *loc. cit.*), blood being obtained from the tail. The animals were starved for about 20 hours before the test. After the fasting blood had been taken, they were given 100 mg. of sugar in 0.5 c.c. to 1.0 c.c. of water with a teat or dropper. Blood was taken from the tail at intervals up to three hours. The results of these tests are shown in Table II. Typical sugar-tolerance curves are given in the Graph.



### Results of glucose-tolerance tests on albino rats.

(a)	Result of sugar-tolerance tests on rats at the end of eleven months of feeding with high carbohydrate diet.	fourteen months.
(b)	" " " " " "	sixteen months.
(c)	" " " " " "	" "

GRAPH.



When 100 mg. of glucose are given to a normal rat, a rise in blood sugar takes place in 30 minutes, reaching a maximum in about 60 minutes. The maximum concentration rarely exceeds 180 mg. per cent and in the course of three hours the blood sugar falls, though not to the fasting level. It takes about five to six hours for the sugar concentration to return to the fasting level (Sankaran and Rajagopal, *loc. cit.*). No evidence of decreased sugar tolerance was observed in rats fed on the experimental diet for 11 months, curves being similar to those seen in normal stock animals, and the fall after one to two hours showing no prolongation. The same result was obtained in three rats surviving for 14 months, and two surviving for 16 months. No rat in the series gave a curve suggesting abnormality in glucose metabolism.

The Graph shows the glucose-tolerance curves of a normal animal, and curves given by an animal (I, II, and III) after 11, 14, and 16 months on the experimental 'high-sugar' diet. None of the latter shows a very high peak or sustained high concentration of sugar. The 'lag' type of curve which might be expected should the animals have developed a condition resembling diabetes mellitus was not obtained.

*Pathological investigation of the pancreas.*—The pancreas of two rats surviving at the end of 16 months were examined by Dr. Radhakrishna Rao who failed to observe any changes in islets of Langerhans.

#### DISCUSSION.

While the research was in progress, Hrubetz (*loc. cit.*) reported an unsuccessful attempt to produce 'idiopathic diabetes' in rats by feeding them on a diet which consisted of a typical stock diet supplemented by 25 per cent of sucrose. In these experiments no sugar-tolerance tests were carried out. Blood-sugar values were determined at intervals for a period of nineteen months.

#### SUMMARY.

An attempt to produce in rats a condition resembling diabetes mellitus by long-continued feeding on a diet based on rice and rich in sucrose proved unsuccessful.

#### ACKNOWLEDGMENTS.

We acknowledge the help of Dr. B. G. Krishnan who devised the experimental diet employed and supervised the feeding of the animals. We are grateful to Major K. R. K. Iyengar, I.M.S., Director, Pasteur Institute of Southern India, Coonoor, for permitting the use of the electrometric apparatus.

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## A CHEMICAL METHOD FOR THE ESTIMATION OF FLAVIN IN FOODSTUFFS.

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It is now established that vitamin B<sub>2</sub> is a complex factor composed of at least two entities, one of these being the factor called vitamin B<sub>6</sub> and the other, flavin. The former is heat stable, the latter more susceptible to heat and fluorescent. At present considerable attention is being given to flavin by research workers. The fact that it is necessary for growth in rats was first demonstrated by Kuhn (1933), György (1933), and their respective colleagues.

The recognition of the importance of flavin as a dietary factor has stimulated the search for rich sources of this material. It has been found in abundance in milk, liver, and yeast. There is, however, a lack of information about the flavin content of many common foodstuffs, for it is difficult to deduce data about flavin content from earlier biological assays of the whole vitamin-B<sub>2</sub> complex. A number of chemical methods for the estimation of flavin have been elaborated (Kuhn *et al.*, *loc. cit.*; Euler *et al.*, 1933, 1934*a* and *b*; Charit and Chaustov, 1935; Pett, 1935*a* and *b*), but while these may work well in the case of particular foodstuffs, they are not suitable for a general survey. There are difficulties in devising a method which may be applied equally to foods rich in protein such as meat and pulses, and to foods rich in fat such as butter, nuts, and edible oil seeds. A further difficulty is that extracts from foodstuffs contain a complex mixture of natural pigments which may hinder the estimation of a particular pigment. Mention must also be made of the observation of György *et al.* (1934) and Euler and Adler (1934*a*), that flavin in milk is present in the free state and easily dialysable, whereas in yeast and liver it is present as a flavo-protein complex, which breaks down under the influence of elevated temperature. In some of the methods referred to above, heat was not employed to break down this complex.

There is thus need for a general chemical method for assaying flavin in all foodstuffs. The present communication describes the standardization and use of such a method, which represents an adaptation of various techniques employed by other workers.

## EXPERIMENTAL.

Chemical procedures for estimating flavin have followed two lines: The pigment is isolated either (a) by precipitation methods depending upon differential solubility in certain solvents or (b) by adsorption on specific adsorbents. It is generally considered that the latter method is more reliable than the former (Wagner-Jauregg, 1934; Nakahara *et al.*, 1934). Hence adsorption technique was adopted in the standardization of a general method.

The technique involving adsorption is divisible into four stages: In stage I flavin is isolated from the test substance by the use of suitable solvents. Stage II concerns the adsorption of flavin from the extract obtained in stage I. In stage III this adsorbate is eluted by a suitable solvent. Finally, in stage IV, a quantitative estimation of flavin is made by comparison against standard lacto-flavin solutions in ultra-violet light.

Certain precautions are necessary for the assay of flavin: First, light must as far as possible be excluded. It is known that visible light has a destructive effect on flavin (Karrer *et al.*, 1935; Supplee *et al.*, 1935), especially in alkaline solutions. Radiations between 3,100 A. U. and 4,800 A. U. are responsible for this effect. Hence, in carrying out the present method of assay light was excluded from start to finish, with the exception of radiations from a red bulb which were used only when absolutely necessary.

A second factor which is of equal importance is temperature. The works of Halliday (1932) and Keenan *et al.* (1935) which relate to the thermal stability of flavin in solution show that even with an acid stabilizer temperatures of 90°C. to 100°C. may cause destruction even in a short period. In the present investigation distillations were carried out under reduced pressure and the temperature of the solution was not allowed to go beyond 50°C. at any stage.

STAGE I.—The best working temperature for extraction must be fairly low and the solution protected by faint acidity. In the present method the temperature is maintained at 37°C. and the extracting liquid is acidified by dilute HCl so that the pH is about 1.0.

*Extracting liquid.*—The flavin solvents most commonly employed are ethyl alcohol, methyl alcohol, and acetone, with varying percentages of water. Acetone, pure or diluted, has not been favoured by many workers. The two alcohols have been used as extractants either absolute or in high concentrations ranging from 60 to 95 per cent (Bourquin and Sherman, 1931; Booher, 1933; Karrer and Schopp, 1934; Charit and Chaustov, 1934; Itter *et al.*, 1935; Pett, *loc. cit.*). The preferential solubility of these alcohols towards vitamin-B<sub>1</sub> and the vitamin-B<sub>2</sub> complexes was investigated by Smith (1933) and Stiebeling and Alleman (1933) who showed that, while ethyl alcohol, with or without acid, takes up vitamin B<sub>1</sub>, leaving vitamin B<sub>2</sub> behind in varying concentration, methyl alcohol takes up vitamin B<sub>2</sub> completely with a certain amount of vitamin B<sub>1</sub>. It thus appears that methyl alcohol is to be preferred as a solvent for vitamin B<sub>2</sub>, and this finds support in the works of Charit and Chaustov (1934) and Karrer and Schopp (*loc. cit.*).

Previous workers have in general used solvents in high concentration. The following experiment was carried out to determine whether lower concentrations could be suitably employed and whether it is possible to make determinations directly on alcohol extracts without adsorption and elution: Weighed quantities

of yeast were treated with 50 c.c. of 20 and 40 per cent methanol as solvent, being incubated for 48 hours at 37°C. The extracts were separated and the residues washed with concentrated methyl alcohol to extract any remaining traces of colour. It was evident that complete extraction could be accomplished by the lower concentrations. The methyl-alcohol solutions were made up to a known volume and aliquots were adsorbed, eluted, and concentrated (stages II and III). This concentrate was used for estimating flavin. It is evident from the following data that under these conditions direct determinations on the alcohol extracts would be vitiated by the presence of extraneous pigments:—

Percentage of methanol.	FLAVIN ESTIMATED BY FLUORESCENT COLOUR IN $\gamma$ PER G.	
	Before adsorption.	After adsorption.
20	1150.0	124.3
40	120.0	104.7

With high concentrations of alcohol and within the limits of solubility, it might be possible (Pett, *loc. cit.*) to carry out determinations directly in the alcohol extracts. Cohen (1935) has, however, produced evidence to show that in aqueous alcoholic extracts of yeast and carrots part of the fluorescence remained after irradiation which would destroy flavin, and also that this residual fluorescence remained unadsorbed on Frankonit.

Twenty per cent methyl alcohol acidified to pH 1.0 with HCl was therefore adopted as the concentration to be used as a solvent. This small degree of acidity is of considerable importance as it stabilizes the flavin in the extract at the distillation temperatures employed, viz., 50°C. (stage II). Further, the acidity, while it brings about precipitation of substances such as proteins, helps to reduce frothing of the solution during subsequent distillation.

*Period of extraction.*—The following experiments were carried out to determine the optimum period of extraction: In these experiments yeast was extracted with 50 c.c. of the solvent as previously described. Flavin determinations were made on samples after 24, 48, and 72 hours. Flavin was estimated before and after adsorption by taking aliquots from stock solutions made up to known volume. The results were as follows:—

Percentage of methanol.	FLAVIN ESTIMATED IN $\gamma$ PER G. BY THE FLUORESCENT COLOUR OF EXTRACT before ADSORPTION AT THE END OF			FLAVIN ESTIMATED IN $\gamma$ PER G. BY THE FLUORESCENT COLOUR OF EXTRACT after ADSORPTION AT THE END OF		
	24 hours.	48 hours.	72 hours.	24 hours.	48 hours.	72 hours.
20	1,435.0	1,148.0	1,158.0	91.5	124.3	109.5
40	772.0	120.9	115.6	136.0	104.7	107.8

The extraction was made at 37°C. The estimations carried out in the extract after adsorption show that with 20 per cent alcohol the extraction was complete after 48 hours, and that with 40 per cent alcohol a period of 24 hours was sufficient.

The use of 20 per cent methyl alcohol, with a 48-hour extraction period at 37°C., was therefore adopted, a procedure which allowed the most economical use of methyl alcohol.

STAGE II. *Adsorption*.—The crude flavin extract obtained in stage I is quantitatively transferred to a distilling flask and the alcohol removed under reduced pressure. The volume is then adjusted to 50 c.c. and the resulting solution used for adsorption.

As regards the adsorbents used in flavin work, there is considerable agreement among workers that Fuller's earth (acid) is a specific adsorbent, and that it adsorbs quantitatively (Booher, 1934; Nakahara *et al.*, *loc. cit.*; Lepkovsky *et al.*, 1935; György *et al.*, *loc. cit.*; Cohen, *loc. cit.*; Karrer *et al.*, *loc. cit.*). There are references to the use of one other adsorbent, i.e., lead sulphide, but this has not found general favour. Although Fuller's earth has been reported by other workers to yield satisfactory results, yet there are certain details in their technique which are time-consuming and of doubtful significance. According to the usual method the solution is left in contact with the adsorbent overnight, and for preparing the adsorbate ready for elution the acid is completely removed by washing. Experiments were conducted to discover the significance of these two procedures. Yeast was used in these experiments also. The flavin content of one sample was determined after allowing it to stand overnight, while in the case of a second sample elution was carried out immediately after adsorption and the determination made directly after eluting the pigment from the acid earth and completely removing the acid. In a third case the procedure followed was the same as in case one but the acid was not removed by washing. The results are given below:—

FLAVIN IN $\gamma$ PER G. ESTIMATED AFTER STANDING OVERNIGHT AND REMOVING THE ACID BY WASHING.	FLAVIN IN $\gamma$ PER G. ESTIMATED WITHOUT THIS INTERVAL AND AFTER WASHING THE ACID COMPLETELY.	FLAVIN IN $\gamma$ PER G. ESTIMATED AFTER STANDING OVERNIGHT BUT WITHOUT WASHING THE ACID.
62.35	57.9	124.3

The above shows that the duration of time allowed for settling has no significance and that washing the acid completely is detrimental. The technique was modified accordingly.

STAGE III. *Elution*.—Removal of the adsorbed pigment from the adsorbent was carried out by eluting with suitable solvents. Alcoholic or aqueous mixtures containing compounds having an alkaline reaction such as ammonia (Kuhn *et al.*, *loc. cit.*), pyridine (Salmon, 1931; Ellinger and Koschura, 1933; György *et al.*, *loc. cit.*; Karrer *et al.*, *loc. cit.*; Stare, 1935; Itter *et al.*, *loc. cit.*) and sodium hydroxide or diethylamine (Lepkovsky *et al.*, *loc. cit.*) have been used for this purpose. Flavin undergoes chemical transformation into compounds having no biological activity under the influence of alkali and elevated temperature.



It has been found in the course of the present work that it is essential that the eluting mixture should be of an acid nature. The results of the flavin determination in yeast given previously show conclusively that fairly strong acidity does not interfere with elution at all. On the other hand, its stabilizing effect is unmistakable. In the present work, therefore, the composition and the nature of the eluting mixture were similar to those of the mixture generally used, i.e., methanol, pyridine, and water in the proportion of 1:1:4, but with the important difference that the medium was kept acid. Shaking in a rocking machine for one hour of the adsorbate with 50 c.c. of the mixture was found to bring about satisfactory elution.

**STAGE IV. Estimation of the flavin in the extract.**—After elution, the earth was removed by centrifuging, the residue washed with methanol, and the washings and centrifugate were distilled under reduced pressure to about 2 c.c. It was then transferred quantitatively into a hand-centrifuge tube by washing thrice with 3 c.c. of acetone, and the solution stirred and centrifuged to remove any insoluble matter. The resulting clear solution was brought to pH 7.0 by the addition of alkali. At this pH the fluorescence is best displayed. The solution was then diluted so that concentration of the pigment was not more than 30 $\gamma$  per c.c. The colour of this solution was then compared against a standard lacto-flavin solution of a similar tint, or against a standard solution of potassium chromate which was in turn standardized against a lacto-flavin solution of known strength. The solutions were put in quartz tubes in a Klett colorimeter and compared in a beam of ultra-violet light obtained from a quartz mercury vapour lamp. To save time all specimens were first examined in natural light against a dark background for green fluorescence. In the absence of fluorescence, estimation in the ultra-violet is unnecessary.

**Summary of the method.**—A suitable quantity of the foodstuff to be tested is finely divided and weighed out carefully into a 250 c.c. stoppered bottle. The quantity of the material is such that its flavin content does not exceed 200 $\gamma$ . The vitamin-B<sub>2</sub> values of common foodstuffs estimated by the biological method could be taken as rough guides in the choice of the quantity. To this weighed quantity of test material 50 c.c. of a solution containing 10 c.c. of methanol\* and 40 c.c. of dilute hydrochloric acid, to render the whole bulk 0.1 N, are added. The contents are shaken well and incubated at 37°C. for 48 hours with occasional shaking. At the end of this period the contents of the bottle are quantitatively transferred into a centrifuge tube and the clear supernatant solution obtained by centrifuging. Addition of 1 c.c. of concentrated HCl at this stage furthers the coagulation of large quantities of protein and of other interfering materials if present. The supernatant solution is transferred into a 250 c.c. distilling flask and the residue in the tube is stirred and centrifuged with methanol until no more colour can be extracted. Usually two or three washings are sufficient. The wash-liquids are transferred to the distilling flask and the alcohol completely removed under reduced pressure, the source of heat being an electric heater. The naked heating coils radiate light energy, and hence the heater has to be covered with asbestos paper thick enough to arrest leakage of light. A dull red light can be used to observe

\* It has been found by actual experiment that technical methanol is as good as pure methanol for this work and it is a great saving to use the technical preparation.

now and then the progress of the distillation. The occasional bubbling of air through the solution prevents any possibility of reduction of the flavin taking place. When the bulk of the solution is about 40 c.c. it is transferred quantitatively into a 250 c.c. stoppered bottle. The solution should be clear at this stage; if not, any suspended matter which separates must be removed by centrifuging. Three grammes of Fuller's earth are weighed out into the solution, followed by the addition of 3 c.c. of concentrated HCl. If during centrifuging any concentrated HCl has been added to assist clarification, the balance of the acid is added at this stage. The bottle is shaken so that the earth is completely wetted. It is then put in a rocking machine and rocked for an hour at moderate speed. At the end of this period the contents of the bottle are quantitatively transferred into a centrifuge tube, and centrifuged for about five minutes. Because of the strong acidity existing in the solution the separation is quite satisfactory. The clear supernatant solution is sometimes coloured due to its containing pigments other than flavin. The supernatant solution is rejected and the Fuller's earth adsorbate transferred into a 250 c.c. stoppered bottle, and a mixture of 50 c.c. of the pyridine-methanol-water mixture added. The bottle is then shaken in the rocking machine for one hour and quantitatively transferred into a clean centrifuge tube and the clear solution separated from the earth by centrifuging. The clear solution is transferred into a 250 c.c. distilling flask and the residue in the centrifuge tube is stirred and centrifuged with methanol until no more colour is extracted. The centrifugate with the washings is distilled under reduced pressure to a volume of about 2 c.c. It is then transferred into a small graduated centrifuge tube, the flask washed repeatedly with small quantities of acetone, and the washings added to the solution in the centrifuge tube; the concentration of the acetone in the mixture should not exceed 60 per cent, as in higher concentrations it ceases to be a good solvent for flavin. The mixture is stirred and centrifuged, and the clear supernatant transferred into a test-tube after reading the volume. Unless some earth settles at the bottom of the centrifuge tube there is no need to wash this residue again. The solution is brought to pH 7.0 by the addition of alkali and centrifuged to remove any insoluble matter which separates at this stage. The clear solution is made up to a known volume and its fluorescent colour is compared against a lacto-flavin solution as already described.

The above method was used to estimate the flavin content of 40 common Indian foodstuffs. The method was found to yield results which are in accordance with other chemical and biological data given in literature. A summary of these results is given in the Table :—

TABLE  
*The flavin content of 40 foodstuffs.*

Foodstuff.	Botanical name.	Moisture (per cent).	Colour of the extract in natural light.	Flavin in $\gamma$ per g. of fresh weight.
Pepper .. ..	<i>Piper nigrum</i>	10.78	Greenish brown	152.0
Dried yeast (Brewer's) ..	—	4.15	Bright greenish yellow.	124.3

TABLE—contd.

Foodstuff.		Botanical name.	Moisture (per cent).	Colour of the extract in natural light.	Flavin in $\gamma$ per g. of fresh weight.
Liver (sheep) ..	—	—	70.78	Bright greenish yellow.	17.0
Skim-milk powder ..	—	—	5.62	„	13.05
Whole milk ..	—	—	89.8	„	12.7 per 10 c.c.
Butter-milk ..	—	—	94.9	„	15.5 per 10 c.c.
Betel leaves ..	<i>Piper betle</i>	—	84.2	Yellowish green	12.6
Ragi ..	<i>Eleusine coracana</i>	—	12.62	Pinkish yellow	11.3
Coriander leaves ..	<i>Coriandrum sativum</i>	—	87.5	Bright yellowish green.	11.2
Beans ..	<i>Phaseolus vulgaris</i>	—	91.0	„	5.66
Green gram (whole) ..	<i>Phaseolus radiatus</i>	—	11.8	Greenish yellow	5.02
Egg white (fowl) ..	—	—	85.25	Bright greenish yellow.	4.96
Lady's fingers ..	<i>Hibiscus esculentus</i>	—	96.4	Greenish yellow	4.54
Tapioca (dry) ..	<i>Manihot utilissima</i>	—	13.89	„	4.17
Ground-nut ..	<i>Arachis hypogæa</i>	—	6.64	Bright greenish yellow.	3.76
Soya beans ..	<i>Glycine hispida</i>	—	18.3	Yellowish green with a pink tint.	3.23
Maize (white) ..	<i>Zea mays</i>	—	16.62	Bright greenish yellow.	3.09
Peas (green and fresh) ..	<i>Pisum sativum</i>	—	49.5	Greenish yellow	2.8
Tomato (ripe) ..	<i>Lycopersicum esculantum</i>	—	94.5	Yellowish green with an orange tint.	2.36
Cabbage (green and fresh)	<i>Brassica oleracea capitata</i>	—	89.48	Greenish yellow	2.15
Tomato (green and fresh)	<i>Lycopersicum esculantum</i>	—	90.88	Yellowish green	1.95
Cashew nut ..	<i>Anacardium occidentale</i>	—	6.71	Light greenish yellow.	1.90
Chillies (green and fresh)	<i>Capsicum annum</i>	—	84.98	„	1.80

TABLE—concl'd.

Foodstuff.	Botanical name.	Moisture (per cent).	Colour of the extract in natural light.	Flavin in $\gamma$ per g. of fresh weight.
Amaranth .. ..	<i>Amaranthus gangeticus</i>	83.39	Greenish yellow	1.56
Rice (parboiled, unmilled)	—	7.09	Yellowish green	1.24
Rice (raw, unmilled) ..	—	12.12	"	1.22
Wheat (whole) ..	—	11.28	"	1.19
Snake gourds ..	<i>Trichosanthes anguina</i>	95.2	Light yellowish green.	1.15
Brinjal .. ..	<i>Solanum melongena</i>	90.86	Greenish yellow	0.91
Rice (milled and polished)	—	12.27	"	0.8
Potato (with peel) ..	<i>Solanum tuberosum</i>	73.1	"	0.43
Butter (fresh) ..	—	13.42	Light yellow	0.08
Carrots .. ..	<i>Daucus carota</i>	87.86	Greenish yellow	Traces.
Bengal gram ..	<i>Cicer arietinum</i>	9.807	"	"
Cambu .. ..	<i>Pennisetum typhoideum</i>	10.26	"	"
Cholam .. ..	<i>Sorghum vulgare</i>	14.36	"	"
Areca-nut .. ..	<i>Areca catechu</i>	39.8	Red with little green tint.	"
Chillies (ripe) ..	<i>Capsicum annum</i>	11.9	Red with tinge green.	Nil.
Dhal (arhar) ..	<i>Cajanus indicus</i>	10.72	Reddish	"
Fish (sea) .. ..	—	80.21	No colour	"

## DISCUSSION.

The Table includes a variety of substances differing considerably in their chemical composition. When these were subjected to the analytical procedure described it was found that differences in the chemical composition did not interfere with the extraction of flavin. Certain of the foodstuffs in the list contain a complex mixture of pigments; on treatment with Fuller's earth, these extraneous pigments were for the most part left unadsorbed. Of the unadsorbed pigments some were fluorescent. The extracts from yeast, tomato, betel leaves, ragi, pepper, peas, cabbage, and carrots possessed such unadsorbable fluorescent colours. This observation of the existence in foodstuffs of non-flavin fluorescent pigments is in conformity with the finding of Cohen (*loc. cit.*) in the case of yeast and carrots.

Fuller's earth, while leaving unadsorbed most of the non-flavin fluorescent pigments, takes up other non-flavin pigments. The pink and scarlet coloured extracts of ragi, dry chillies, areca-nut, and dhal arhar were examples of such pigments. These pigments do not possess any fluorescence either in natural light or in the ultra-violet beam. From this it follows that methods of estimation of flavin depending upon its fluorescence in the ultra-violet rays are preferable to those in which the colour in natural light is estimated. In the latter, the extraneous non-flavin pigments adsorbed by the Fuller's earth interfere, especially when they are strong.

The flavin-content figures presented in the Table show fairly close agreement with values reported by other workers for similar foodstuffs obtained by biological assays. A reference to the Table shows that pepper, yeast, and animal foods are the best sources for this vitamin. Vegetable foods with the exception of pepper and yeast are inferior to animal foods, and some are very poor sources. Pepper, though in small quantity, finds daily use in the Indian dietary and the finding that it is rich in flavin is of interest. It is, however, necessary to obtain confirmatory evidence of the richness of pepper in flavin by biological experiments. These experiments are in progress.

Flavin has oxidizing properties, while vitamin C is a reducing agent. It appears that in general rich sources of flavin are deficient in vitamin C, and vice versa. Foods with a high vitamin-C content, such as tomato, cabbage, green chillies, amaranth, and potatoes, are poor in flavin. It seems possible that there may be an inverse relation between the flavin and ascorbic-acid content of foodstuffs, due to their nature.

#### SUMMARY.

1. A method has been standardized for the chemical assay of flavin in foodstuffs.
2. The results of the analyses of 40 foodstuffs are presented. They are in fairly close agreement with the results of the biological assays of flavin and vitamin B<sub>2</sub>.

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## THE EFFECT OF SKIMMED MILK, SOYA BEAN, AND OTHER FOODS IN SUPPLEMENTING TYPICAL INDIAN DIETS.

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MILK is a foodstuff of particular importance in a country in which the diet is largely vegetarian and the consumption of meat small. A small percentage of the population abstains from flesh food and eggs from religious conviction; the majority have, however, no objection to eating eggs and certain kinds of meat, but are prevented from doing so by their poverty and the scarcity of such foods. The average Indian diet is thus relatively deficient in proteins of high biological value and the food factors, e.g., vitamin B<sub>2</sub>, which are usually found in association with proteins of this nature. The value of milk as food for children is recognized even by the illiterate, but over great areas of the country whole milk is scanty and beyond the reach of the poor. A recent village diet survey (Aykroyd and Krishnan, 1937a) demonstrated the small consumption of milk in typical South Indian village communities.

In the course of an investigation in residential institutions for children (Aykroyd and Krishnan, 1937b), we were faced with the practical problem of improving diet schedules without great increase in cost. The cost of food supplied in Mission hostels, etc., often does not exceed Rs. 3 per child per month, and whole milk cannot be supplied at this level of expenditure. A cheaper substitute is essential.

The present paper describes an investigation of the value of skimmed or separated milk as a supplement to the diet of school children. The high protein and fat content of soya bean suggested that this legume might make good some of the deficiencies of typical South Indian diets, and its supplementary value was investigated in the same way. Parallel experiments with these and other foods were carried out on rats.

## EXPERIMENTS IN BOARDING SCHOOLS.

A residential hostel (hostel 1) supported by a Christian Mission in a South Indian town, containing 122 boys aged 7 to 19, the majority between 11 and 15, was chosen as the venue of an investigation. The pupils were village boys largely belonging to the so-called 'depressed' classes. The missionary in charge offered full co-operation. The diet supplied in this hostel was fairly typical of diets consumed in many parts of India. Precise data about the foods purchased and consumed during a single typical month previous to the investigation were obtained, and intake per consumption unit or 'man value' per day calculated, the earlier International scale of family co-efficients (League of Nations Health Organization, 1932) being used. The composition of the diet per consumption unit per day was roughly as follows :—

TABLE I.

*Composition of the diet in hostel 1.*

Food.	Per consumption unit per day in oz.
Cambu ( <i>Pennisetum typhoideum</i> ) .. ..	9.0
Milled parboiled rice .. ..	10.0
Dhal arhar ( <i>Cajanus indicus</i> ) .. ..	2.7
Leafy vegetables .. ..	0.3
Non-leafy vegetables .. ..	2.0
Coriander seeds .. ..	0.2
Tamarind .. ..	0.2
Plantains .. ..	0.5
Gingelly oil .. ..	0.1
Ground-nut .. ..	0.3
Coco-nut .. ..	0.2
Mutton .. ..	0.5
Protein (g.) .. 80	Calcium (g.) .. 0.28
Fat (g.) .. 25	Phosphorus (g.) .. 1.70
Carbohydrate (g.) 451	Iron (mg.) .. 42.5
Calories 2,352	International vitamin A units, roughly .. 1,200



While this diet *resembles* that consumed by the poorer classes in many parts of India, it is on the whole rather more varied and abundant. The boys were in a fairly good 'state of nutrition' and the incidence of food deficiency disease, as evidenced by xerophthalmia, phrynoderma, and stomatitis, was comparatively low. Their condition compared well with that of boys in the general population and of boys examined in other institutions in which the whole of the cereal ration was composed of milled rice.

The boys live in six houses, each of which contains boys of similar age distribution. The school was divided at random into two groups, A and B, with three houses in each. The age distribution in the groups is given in Table II :—

TABLE II.

*Age distribution of groups A and B (hostel 1).*

Age.	Group A.	Group B.
7	1	1
8	0	2
9	0	4
10	1	4
11	5	8
12	16	10
13	8	9
14	15	4
15	9	11
16	2	4
17	4	1
18	1	1
19	0	0
20	0	0
21	1	0
TOTALS ..	63	59

It will be seen that there is fair correspondence in age distribution, the majority of boys in both groups being from 11 to 15 years of age. The mean age in each

was about 12 years. It must, however, be observed that the recorded age of school children of the poorer classes in India is subject to a considerable margin of error.

Group A was given 1·0 oz. of skimmed milk powder (New Zealand, 'Acorn' Brand, Spray Process) daily for three months as an addition to the ordinary hostel diet. The taking of milk was supervised by the missionary in charge, who took a great interest in the experiment. It was given in liquid form, roughly eight parts of water being added for one part of powder to re-constitute liquid milk. The boys in group B received no milk supplement, but a little more cambu (millet) so that calorie intake should be roughly equal in both groups.

The boys were weighed and measured at the beginning and end of the first three months' period, an Avery's lever balance being used. Height and weight increases were as follows:—

HOSTEL 1. GROUP A (receiving milk)—

Average increase in weight 4·77 lb.

„ „ „ height 0·61 inch.

HOSTEL 1. GROUP B (not receiving milk)—

Average increase in weight 2·13 lb.

„ „ „ height 0·35 inch.

At the end of the first experimental period the school went on holiday for three weeks. During the holiday both groups put on weight, the increase being greater in the group which had received milk. When the boys returned to school, they were weighed and measured again and conditions in the two groups were reversed, group B now receiving 8·0 oz. of liquid skimmed milk, while group A went without. Average height and weight increments at the end of the second experimental period were as follows:—

HOSTEL 1. GROUP B (receiving milk)—

Average increase in weight 3·07 lb.

„ „ „ height 0·69 inch.

HOSTEL 1. GROUP A (not receiving milk)—

Average increase in weight 1·10 lb.

„ „ „ height 0·43 inch.

The first experimental period lasted from 15th June, 1936 to 21st September, 1936 (14 weeks); the second from 7th October, 1936 to 18th December, 1936 (10½ weeks). There was thus a fair correspondence between the weight increases in the groups receiving and not receiving milk in the two experimental periods. In each period the milk-fed group showed much larger average increase in weight and height than the control group.

The differences between the height and weight increases in the groups in both the experimental periods are 'statistically significant' (see Appendix).

No change was made in the 'basal' diet during the experiment. The addition of milk was the only variable.

Experiments in two other children's hostels (hostels 2 and 3), containing 35 girls and 32 boys respectively, provided confirmatory evidence of the nutritive

value of skimmed milk. The majority of children were aged 10 to 16. The diets of these groups were in general similar to that described in Table I, except that the only cereal provided was parboiled milled rice. These diets have been fully described in a previous paper (Aykroyd and Krishnan, 1936b); in the girls' hostel the percentage of total calories obtained from rice was 78; in the boys, 76. Milled rice is of lower nutritive value than millet, and the children in these hostels were in a poorer general condition than those in the first hostel in which a fair proportion of cereal intake was in the form of cambu. Of the girls 70 per cent and of the boys 50 per cent were suffering from stomatitis due to vitamin-B<sub>2</sub> deficiency, and Bitot's spots and phrynoderma were observed among the children, the latter being present in a high percentage. Skimmed milk was given for the treatment of stomatitis, and proved rapidly effective.

In the girls' hostel (hostel 2) 1·5 oz. of skimmed milk powder (12·0 oz. of re-constituted liquid milk) were given daily. The average increase in weight during a period of three months was 4·8 lb.; of height 0·80 inch. In a neighbouring girls' hostel, where the diet was roughly the same, but no skimmed milk was given, average weight and height increases were 0·8 lb. and 0·56 inch respectively.

During a second period of three months' feeding with skimmed milk, average increases in weight and height were 1·8 lb. and 0·53 inch respectively.

In the boys' hostel (hostel 3) we had accurate weight records taken three months before the milk was given. Further weight and height records were obtained at the beginning of the 'milk' period, and again after milk had been given for three and six months. One oz. of milk powder (8·0 oz. of liquid milk) were given. During the first period of three months (non-milk), average increase in weight was 0·84 lb. During the second period of three months (milk supplement) average increase of weight was 4·57 lb.; of height 0·67 inch. These increases correspond to those observed in group A, hostel 1, during the first experimental period, and also to those recorded in hostel 2, during the first three months of milk feeding.

Average weight and height increases in hostel 3, during a second period of three months' feeding with skimmed milk, were 1·6 lb. and 0·59 inch respectively.

#### THE EFFECT OF SKIMMED MILK ON GENERAL HEALTH AND STATE OF NUTRITION.

In all three hostels an improvement in general health was observed in the milk-fed groups. The improvement was very obvious, both to ourselves and the hostel superintendents, particularly in hostels 2 and 3. The children appeared to become 'sleeker', and to show higher spirits and enhanced vitality. There was an unquestionable fall in the incidence of minor ailments. The Lady Superintendent of hostel 2 wrote as follows:—

'From the Matron and attendance register, I learn that there has been a considerable drop in the usual incidence of minor sickness (during the period of milk feeding). The doctor at our little local hospital informs me that there has been less demand for 'fever mixture' than ever before in our history and instead of the almost daily trickle of patients from our girls' hostel, visits to the hospital have been very much more infrequent.'

Actually the improvement in general condition was as striking a feature of these experiments as the enhancement of growth, though it cannot, so to speak, be recorded quantitatively. It was particularly evident in the groups which received milk as a supplement to diets largely composed of milled rice; we may say without exaggeration that a remarkable transformation in the appearance of these children was evident.

We have already recorded the cure of 'stomatitis' by skimmed milk. It is of interest to note that skimmed-milk feeding has a curative effect on 'phrynoderma'. The nature and ætiology of this condition has been fully described in previous papers from the Laboratories (Aykroyd and Rajagopal, 1936; Radhakrishna Rao, 1937). Very marked improvement was observed in cases of phrynoderma in hostels 2 and 3 towards the end of the six months' period of milk feeding. This skin condition has been ascribed to vitamin-A deficiency, but skimmed milk is devoid of vitamin A and carotene. It is conceivable that the consumption of skimmed milk increases assimilation by improving the nutrition of the body cells, so that whatever vitamin A is present in the diet is fully absorbed and utilized.

The giving of skimmed milk produced a rapid increase in weight over a period of three months. When the giving of milk was prolonged for a further period, the rate of increase in weight slackened. This is to be expected. While the milk was being consumed the general condition of the children remained excellent, and there was no recrudescence of stomatitis in hostels 2 and 3.

#### THE EFFECT OF A SOYA BEAN SUPPLEMENT.

A similar experiment to that carried out in hostel 1 was organized in another boarding school (hostel 4), 1.5 oz. of soya bean being given instead of skimmed milk. Hostel 4 contained 75 girls, mostly between 10 and 15; one group of 38 girls was given roughly 1.5 oz. of soya bean daily for 20 weeks, the other group of 37 receiving some extra rice so that calorie intake was similar in both groups. The groups were a random selection of similar age composition. The beans were given 'whole', being cooked until soft. They were well liked by the children.

The diet in hostel 4 resembled that supplied in the other hostels. Seventy per cent of total calories were derived from cereals, milled parboiled rice, home-pounded raw rice, and millet (ragi: *Eleusine coracana*) being consumed in roughly equal proportions. The diet contained very small quantities of vegetables and no milk. A little liver was consumed twice a week. The girls were in a poor general condition, but relatively free from symptoms of deficiency disease. Their 'state of nutrition' was slightly better than that of the girls in hostel 2, which may be ascribed to the fact that, whereas in hostel 2 the cereal ration was entirely composed of milled rice, home-pounded rice, and millet, in addition to milled rice, were consumed in hostel 4.

Analysis of the diet supplied in a typical month previous to the investigation did not indicate any serious deficiency in energy value.

Weight records of the two groups, taken at the beginning and end of the 20 weeks' experiment, are available; height records only for a period of 8 weeks

at the end of the experimental period. It was found that both groups lost weight. Results were as follows:—

GROUP RECEIVING SOYA BEAN SUPPLEMENT OF 1·5 OZ. PER DAY—

Average decrease in weight (20 weeks) 2·37 lb.

„ increase in height (8 weeks) 0·46 inch.

GROUP NOT RECEIVING SOYA BEAN SUPPLEMENT—

Average decrease in weight (20 weeks) 0·62 lb.

„ increase in height (8 weeks) 0·45 inch.

The fact that both groups lost weight suggests that the diet may have been insufficient in quantity. It is clear that the addition of soya bean in the circumstances conferred no advantage. The greater loss of weight in the soya bean group may probably be ascribed to the fact that this legume is poorly assimilated; the girls in the control group, who received extra rice in lieu of soya bean, assimilated a larger quantity of food.

Particular emphasis must be laid on the fact that *no improvement*, resembling that brought about by milk, was observed in the general condition of the children consuming soya bean. We have further observed, in another series of experiments, that soya bean is without effect on stomatitis.

The differences in the weight changes in the two groups are 'statistically significant'; as regards height, there is no 'significant' difference.

A statistical note by Professor Madhava on the height-weight data obtained in these experiments is given at the end of the paper.

COST OF SUPPLEMENTS.

During the experimental periods the cost of the diet supplied in hostel 1 averaged Rs. 2-13 per boy per month. To supply 1·0 oz. of skimmed milk powder daily costs 12 annas per month, a very appreciable addition to the budgets of residential institutions run at a minimum level of expenditure. In hostels 2 and 3 the relative increase in cost was roughly similar. One and a half ounces of soya bean daily costs about 5 to 6 annas per month.

ANIMAL EXPERIMENTS.

There are three ways of testing the nutritive value of a diet: first, its chemical composition and vitamin content can be worked out by reference to tables of food values and compared with 'generally accepted standards'; secondly, it can be fed to laboratory animals and its effect on the rate of growth, etc., observed; thirdly, it can be tested by its effect on the development and health of human beings. The last method is the most satisfactory, the first the least. The second method, while not without defect, nevertheless provides useful information about the nutritive value of diets. The value of skimmed milk, soya bean, and a number of other foods as additions to a typical South Indian diet has been tested by growth experiments on rats.

The following diet, known in the laboratory as the 'poor Madrassi diet', is substantially the same as that described in McCarrison's (1931) 'Food' as the 'poor Hindu family diet':—

				Oz.
Raw polished rice	..	..	..	21·00
Dhal arhar	..	..	..	0·70
Black gram	..	..	..	0·70
Brinjal	..	..	..	1·00
Amaranth leaves	..	..	..	0·50
Raw plantain	..	..	..	0·50
Gingelly oil	..	..	..	0·10
Coco-nut	..	..	..	0·05
Meat	..	..	..	0·06

The quantities given correspond roughly to adult daily intake.

This diet, mixed and fed in the proportions indicated, formed the basal diet in these experiments. In testing most of the supplements, 1·5 oz. (42 g.) of each were added to the basal diet and the resulting mixture given. This quantity roughly corresponds to the amounts of skimmed milk powder and soya bean given to the school children. Certain supplements were also fed at a higher level of intake, e.g., soya bean, dhal arhar, and green leafy vegetables.

The method followed was the same as that previously employed in testing a number of 'cheap balanced diets' costing Rs. 4 to Rs. 5 per adult per month (Aykroyd and Krishnan, 1936a). Groups of young rats, 40 g. to 50 g. in weight, were fed on the test diets for 10 weeks. Experience has shown that the 'cheap balanced diets' described in the earlier paper are not cheap enough to be recommended for children's institutions, etc., in India. We wished therefore to obtain data about the value of single supplements to a typical Indian diet which would enable us to suggest dietary alterations more within the means of the population.

Average increase in weight during 10 weeks' feeding of the various test diets is given in Table III. Various weight curves are shown in the Graph.

TABLE III.

Diet.	Supplement.	Average weekly increase in weight (g.).
	<i>Plus.</i>	
Poor Madrassi diet.	No addition .. .. .	2·8
	1·5 oz. skimmed milk powder .. ..	7·4
	1·5 „ whole milk powder (Klim) .. ..	6·7
	1·5 „ egg (yolk and white) .. ..	6·8
	1·5 „ egg-yolk .. ..	6·5

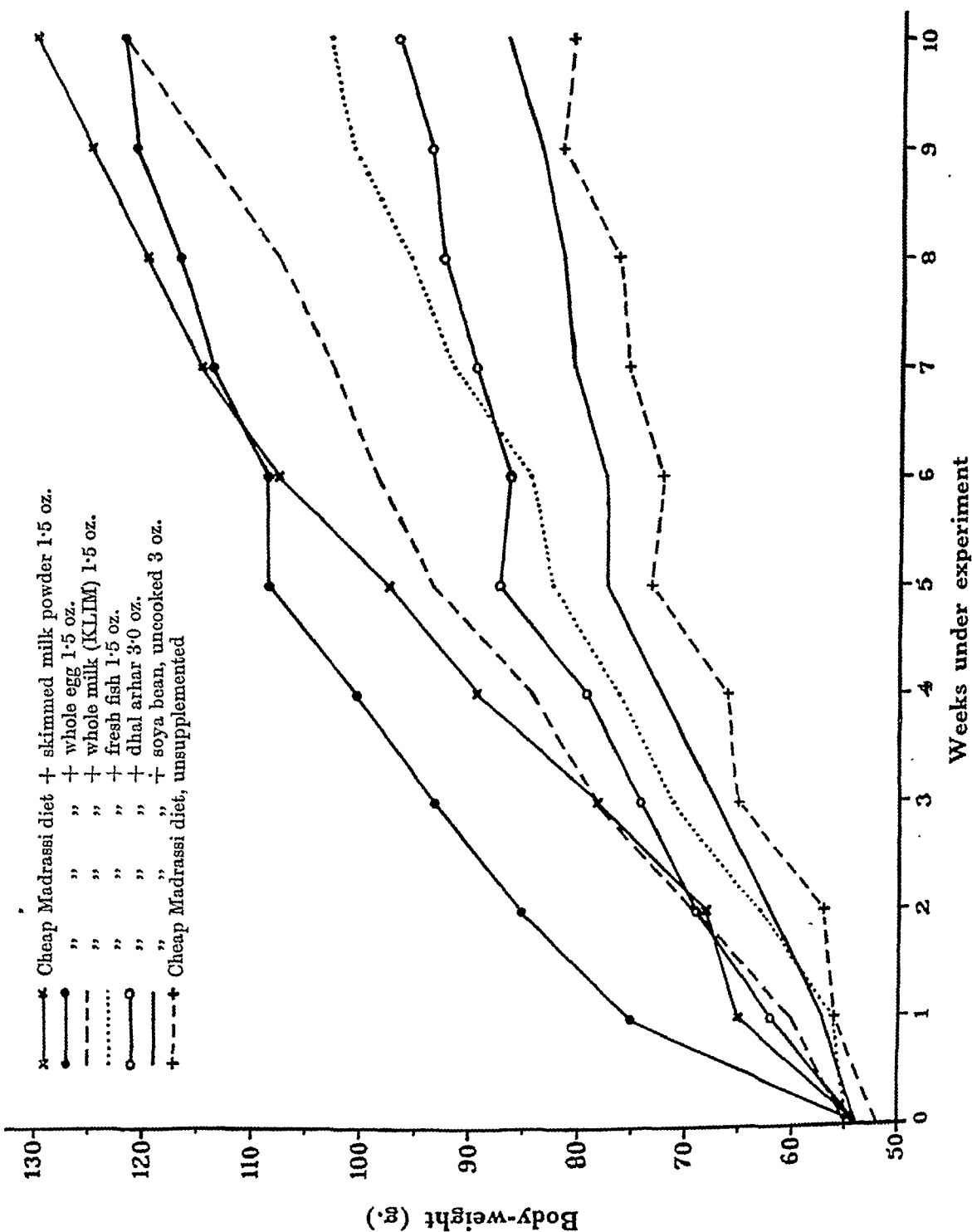
TABLE III—*concl'd.*

Diet.	Supplement.	Average weekly increase in weight (g.).
	<i>Plus.</i>	
Poor Madrassi diet.	1.5 oz. egg-white .. .. .	3.0
	1.5 „ fresh fish .. .. .	4.8
	4.0 „ green leafy vegetables* .. .. .	4.7
	3.0 „ dhal arhar ( <i>Cajanus indicus</i> ) .. .. .	4.3
	1.5 „ black gram ( <i>Phasolus mungo</i> ) .. .. .	3.0
	3.0 „ soya bean (uncooked) .. .. .	3.3
	1.5 „ soya bean (uncooked) .. .. .	3.6
	1.5 „ soya bean (cooked) .. .. .	3.6
	1.5 „ red-palm oil .. .. .	2.9
	1.5 „ gingelly oil .. .. .	2.2
	1.5 „ peeled ground-nuts .. .. .	3.8

\* Amaranth leaves .. .. 2.0 oz.  
 Drumstick leaves .. .. 1.0 „  
 Spinach .. .. 1.0 „

On the Coonoor stock diet, consisting of whole-wheat chapattis smeared with butter, raw cabbage, raw carrots, sprouted Bengal gram (*Cicer arietinum*), cow's milk *ad lib.*, and meat twice a week, the average weekly increase in weight of similar groups of rats was found to be 10.5 grammes. On the 'poor Madrassi diet', the animals showed very poor growth. Even the diet supplemented by milk did not produce anything like an 'optimum' rate of growth. The improvement in the nutritive value for rats of the basal diet by the addition of milk, egg-yolk, and whole egg was, however, clearly evident.

The addition of 1.5 oz. and 3.0 oz. of uncooked soya bean produced no marked supplementary effect. Cooked soya bean was tried as a supplement because of the recent observation of Hayward, Steenbock and Bohstedt (1936), that the nutritive value of soya bean proteins was doubled by the application of high degrees of heat (140°C. to 150°C. for 2½ minutes; 121°C. for 90 minutes), which apparently 'caused some essential protein fraction, unavailable in the raw soya bean, to become available for absorption and metabolic use'. The cooked soya bean used in the rat growth experiment was cooked for two hours. Apparently this degree of heat had no marked effect on its value as a supplement.



Showing increase of weight of groups of rats on the 'Madrassi diet' alone and the same diet variously supplemented.



The additions which proved of most value in enhancing growth were milk powder and eggs, foods which contain proteins of high biological value and are rich in one or more factors present in the vitamin-B<sub>2</sub> complex. The group receiving whole egg showed the most rapid increases during the first five weeks. The diet containing skimmed milk powder produced more rapid increase in weight than the diet containing whole milk powder. This may be ascribed to the fact that a given quantity of dried skimmed milk, being devoid of fat, contains more protein and other factors than a similar quantity of dried whole milk.

Fresh fish, in the quantity fed, was less effective than milk powder and eggs, probably because it is a less concentrated food. Of the pulses tested, dhal arhar appeared to be the most efficient. It is to be observed that the basal diet contained a certain quantity of pulses. The animals on diets containing supplements of leafy vegetables, ground-nuts, black gram, gingelly oil, and red-palm oil showed very little better growth than rats on the 'Madrassi diet' alone. Red-palm oil is a rich source of carotene. Coonoor rats fed on the rich stock diet are well stored with vitamin A, and will grow fairly well for 10 weeks on a diet deficient in vitamin A but otherwise complete. Hence the vitamin-A content of the supplements was not a matter of importance, and their effect on growth was related to their content of other food factors. For this reason rat growth experiments as described may not demonstrate the true value of supplements to a typical South Indian diet, since vitamin-A deficiency is common in South India. But the value of skimmed milk in improving the 'state of nutrition' of school children shows that lack of vitamin A is not the only important defect in South Indian diets.

There is a close parallelism between the experiments with school children and the rat growth experiments. We feel justified in using rat growth experiments as an *indication* of what additions to human diets are likely to prove most valuable.

#### DISCUSSION.

Experiments demonstrating the value of 'extra' milk in enhancing growth and improving general condition have been carried out in a number of countries. In certain of these experiments skimmed milk was given (Leighton and Clarke, 1929; Orr and Leighton, 1929). A summarized account of various 'extra' milk experiments on school children will be found in a recent League of Nations Report (Burnet and Aykroyd, 1935). In all such experiments it has been observed that children receiving 'extra' milk show greater height and weight increases than 'controls' on a similar diet without the 'extra' milk, and that the milk-fed group displays a marked improvement in general condition.

In India an 'extra' milk experiment has been carried out among school children in Simla (Crichton, 1936). One pound of whole milk was given to a selected group of malnourished day-school children, and their weight and height increments compared with those of children not receiving milk. Increases in height and weight in the Simla children receiving and not receiving milk corresponded fairly closely to those observed in the various groups in the present experiments with skimmed milk, and a similar improvement in general condition and a reduction of minor ailments were observed.

Previous to the present experiments, however, it could not be assumed that skimmed milk would prove a valuable supplement to Indian diets containing a high

proportion of cereals and relatively deficient in 'protective' foods. The diets in the hostels in which skimmed milk was given, corresponding to those consumed by the general population, appeared to be poorly supplied with vitamin A. It seemed possible that no improvement in 'state of nutrition' by a single dietary supplement would be obtained unless that supplement was a good source of vitamin A (e.g., whole milk). It is now clear, however, that an important defect in diets of the type described is their lack of one or more of the food factors contained in skimmed milk: proteins of high biological value; vitamin B<sub>2</sub>; possibly calcium. This finding is of far-reaching importance in connection with the whole problem of malnutrition in India and the East.

The all-round improvement which takes place in South Indian children when skimmed milk is given regularly for a few months is an indication that average Indian children, fed on a largely cereal diet lacking 'protective' foods, are in a poor 'state of nutrition', with their physical potentialities largely undeveloped.

One and a half ounces of soya bean daily supply about 20 grammes of protein and 9 grammes of fat, while one ounce of skimmed milk powder contains about 11 grammes of protein and no fat. The fact that soya bean does not appear to 'supplement' South Indian diets may perhaps be ascribed to the low biological value of its proteins. In China and Japan considerable use is made of fermented soya bean products, but the whole bean as such appears to be rarely consumed. It is possible that these soya bean products are of higher nutritive value than the whole bean itself. But the present experiments suggest that there is little purpose in encouraging the wider use of soya bean in India and that the present widespread enthusiasm for this legume is unjustified. From the rat growth experiments it appears that it has no advantage in nutritive value over certain pulses which have long formed part of the Indian dietary.

#### THE POPULARIZATION OF SKIMMED MILK PRODUCTS IN INDIA.

Skimmed milk is the cheapest food so far discovered which effectively 'supplements' the South Indian diet. At average South Indian prices, re-constituted skimmed milk, as used in these experiments, costs about one-third the price of similar quantity of whole milk. In many parts of India a regular supply of unadulterated whole milk, for use in institutions, etc., cannot be obtained without great expense of time and trouble, or cannot be obtained at all.

At present milk imports to India other than from the United Kingdom carry an *ad valorem* import duty of 30 per cent. Duty on imports from the United Kingdom is 20 per cent. Even the cheap skimmed milk product employed in our investigation is at present somewhat too dear for daily use in residential institutions for children in India. It is, however, *almost* within the reach of such institutions. If the 30 per cent duty were removed it could be included in the diet schedules of the numerous children's homes, etc., existing throughout the country, and its more general use would be thereby furthered.

Public health authorities are inclined to oppose the popularization of skimmed milk products on the grounds that these are dangerous to infants. It is, of course, well known that skimmed milk is not suitable for use as the *main* food of infants, unless supplemented by some source of vitamin A, e.g., cod-liver oil or red-palm oil. It may, however, be used with advantage to supplement the diets of young children

past infancy when such diets are largely based on cereals and contain few vegetables and no animal protein; skimmed milk in such circumstances is much better than no milk at all. Its value as a food for older children has been amply demonstrated by the present experiments. It should be possible for public health authorities to take steps whereby the use of skimmed milk by older children should be encouraged and the danger of its being used as the exclusive food of infants minimized. The supply of skimmed milk powder in large containers to day schools and residential institutions would not necessarily enhance its popularity as an infant food.

It is, of course, advisable that when skimmed milk is added to the diet of children attention should simultaneously be given to vitamin-A intake; this may be increased by giving cod-liver oil or raising the consumption of green leafy vegetables.

Unquestionably, it is preferable that Indian children should consume whole milk locally produced. At present, however, in many parts of the country, it is a case of imported milk or no milk at all. It remains questionable whether, in many areas, demand for milk can create an adequate local supply. It will be some time before cheap standard *skimmed* milk products, locally produced, become available in quantity in India.

#### SUMMARY.

1. The addition of liquid skimmed milk, re-constituted from powder, to the diet of children in residential hostels in South India was found to produce an acceleration of growth and a marked improvement in general condition. The diets consumed by the experimental groups were typical South Indian diets.

2. The addition of an amount of soya bean supplying rather more protein did not bring about the same effect.

3. Parallel experiments were carried out on rats. The growth of rats on a typical South Indian diet was enhanced by the addition to the diet of small quantities of milk, whole or skimmed, and eggs. Soya bean was found to be a relatively poor supplement. The addition of green leafy vegetables, dhal arhar, black gram, red-palm oil, gingelly oil, and ground-nuts, did not markedly increase the nutritive value of the basal diet, as assessed by rate of increase in body-weight.

4. The most serious defect in South Indian diets is their deficiency of one or more of the food factors contained in skimmed milk. It is at present impossible to say which of these factors is the most important.

5. The problem of popularizing the use of skimmed milk products in India is discussed.

#### ACKNOWLEDGMENTS.

We wish to express our gratitude to the various superintendents of children's homes who enthusiastically co-operated with us in these investigations.

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# APPENDIX.

## STATISTICAL NOTE

ON THE HEIGHT-WEIGHT DATA OBTAINED IN SKIMMED MILK  
AND SOYA BEAN EXPERIMENTS.

BY

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THE chief conclusion, which is confirmed by the repeated experiment, is that although the two groups (M, receiving milk; N-M, not receiving milk) are not differentiated in respect of age, initial height or initial weight, they show that the effect of introducing skimmed milk was to enhance the increases in weight and height during the progress of the experiments. The sample means in each of the groups in each experiment, with the estimated ranges outside which the true mean may not lie, on the theory of probability, except (a) in five samples out of 100, and (b) in one sample out of 100, are given below. From these figures it is clear that the above conclusion is fully justified.

### *Weight increments (lb.).*

	NO MILK (N-M).		MILK (M).	
	First experiment.	Second experiment.	First experiment.	Second experiment.
Sample mean ..	2.13	1.10	4.77	3.07
Estimated range outside which the true mean may not lie except in			Extent by which the sample mean (M) exceeds the maximum range of variation.	
(a) five samples out of 100 ..	1.51 to 2.74	0.53 to 1.67	2.03	1.40
(b) one sample out of 100 ..	1.32 to 2.94	0.35 to 1.85	1.83	1.22

*Height increments (inches).*

	NO MILK (N-M).		MILK (M).	
	First experiment.	Second experiment.	First experiment.	Second experiment.
Sample mean ..	0.35	0.43	0.61	0.69
Estimated range outside which the true mean may not lie except in			Extent by which the sample mean (M) exceeds the maximum range of variation.	
(a) five samples out of 100 ..	0.27 to 0.43	0.35 to 0.51	0.18	0.18
(b) one sample out of 100 ..	0.25 to 0.45	0.33 to 0.53	0.16	0.16

Records of (a) weight, (b) height at the commencement of the two experimental periods, (c) alteration in weight, and (d) alteration in height at the end of these periods in the M and N-M groups are given in Tables A, B, C, and D in the form of frequency distributions. At the foot of the tables the statistical constants obtained by analysing these data are set out in detail. These include, besides the mean and standard deviation with their respective standard errors, the results of the test for 'goodness of fit' of the distribution of each of the characters in the two groups. Values of  $r$  and  $\eta$ , with the nature of regression and the significance in respect of variance, are also included.

The analysis serves to show that the gain in weight is not in any significant manner associated with initial height and initial weight, or even with gain in height. To associate gain in weight with the introduction or non-introduction of skimmed milk, the method of biserial correlation was applied to the distributions in Table C distinguishing the M and N-M groups. The biserial  $r$ 's are

First experiment:  $0.5530 \pm 0.09$

Second experiment:  $0.4971 \pm 0.09$

It should therefore be possible to conclude that the association (of the order 0.5) is large; it is also consistent with regard to its standard error (of the order 0.09). The test of 'goodness of fit' also confirms the effect of administering skimmed milk in increasing weight.

TABLE A.

*Frequency distribution of weight at commencement of experiment (hostel 1).*

Original weight (lb.).	FIRST EXPERIMENT.		SECOND EXPERIMENT.			FIRST EXPERIMENT.		SECOND EXPERIMENT.	
	Milk (M).	No milk (N-M).	Milk (M).	No milk (N-M).		M group (63).	N-M group (59).	M group (58).	N-M group (60).
30-39.9 ..	1	1	1	..	Mean with standard error	70.08 $\pm$ 2.37	68.73 $\pm$ 2.55	71.00 $\pm$ 2.66	78.80 $\pm$ 2.26
40- ..	7	12	7	..	S. D. with standard error	18.81 $\pm$ 1.08	19.56 $\pm$ 1.80	20.23 $\pm$ 1.86	17.53 $\pm$ 1.53
50- ..	17	11	14	9	<i>Test for goodness of fit—</i> (n) Degrees of freedom $\chi^2$ P Significance	9 8.09 0.73 Not significant.	9 13.62 0.14 Not significant.	9 13.62 0.14 Not significant.	9 13.62 0.14 Not significant.
60- ..	9	7	7	14					
70- ..	9	9	9	11					
80- ..	8	11	7	8					
90- ..	8	5	9	10					
100- ..	3	2	2	6	<i>Correlation with alteration in weight—</i> $r$ $\eta$ Variance Regression	Not significant.	Not significant.	Not significant.	Not significant.
110- ..	1	0	1	1					
120- ..	0	1	1	1					
TOTALS ..	63	59	58	60					
						-0.01 $\pm$ 0.13	0.06 $\pm$ 0.13	-0.22 $\pm$ 0.13	-0.05 $\pm$ 0.13
						0.4785	0.1852	0.3644	0.2985
						Significant.	Not significant.	Not significant.	Not significant.
						Non-linear.	Linear.	Linear.	Linear.

TABLE B.

Frequency distribution of height at commencement of experiment (hostel 1).

Original height (inches).	FIRST EXPERIMENT.		SECOND EXPERIMENT.		FIRST EXPERIMENT.		SECOND EXPERIMENT.	
	Milk (M).	No milk (N-M).	Milk (M).	No milk (N-M).	M group (63).	N-M group (59).	M group (58).	N-M group (60).
45-46.9	..	3	2	..	56.95 $\pm$ 0.60	56.17 $\pm$ 0.71	56.00 $\pm$ 0.72	57.77 $\pm$ 0.62
47-	3	2	4	1	4.74 $\pm$ 0.42	5.42 $\pm$ 0.50	5.45 $\pm$ 0.50	4.82 $\pm$ 0.43
49-	4	0	6	5	<i>Test for goodness of fit—</i>			
51-	5	12	11	3	(n) Degrees of freedom			
53-	13	0	2	11	$\chi^2$			
55-	9	8	8	9	P			
57	7	9	3	8	Significance			
59	6	6	9	2	<i>Correlation with alteration in weight—</i>			
61	9	5	7	11	$r$			
63	4	6	4	6	$\eta$			
65	3	2	2	4	Variance			
					Regression			
TOTALS ..	63	59	58	60	0.04 $\pm$ 0.13	0.14 $\pm$ 0.13	-0.12 $\pm$ 0.13	-0.03 $\pm$ 0.13
					0.3421	0.2958	0.2059	0.2377
					Not significant.	Not significant.	Not significant.	Not significant.
					Linear.	Linear.	Linear.	Linear.



TABLE C.

*Frequency distribution of alteration in weight during the experiment (hostel I).*

Alteration in weight (mid-values).	FIRST EXPERIMENT.		SECOND EXPERIMENT.	Mean with standard error S. D. with standard error <i>Test for goodness of fit—</i> (n) Degrees of freedom $\chi^2$ $P$ Significance	FIRST EXPERIMENT.		SECOND EXPERIMENT.	
	Milk (M).	No milk (N-M).	Milk (M).		No milk (N-M).	M group (63).	N-M group (59).	M group (58).
<i>Lb. gained.</i>								
+	2	..	..	..	4.77 $\pm$ 0.37	2.13 $\pm$ 0.31	3.07 $\pm$ 0.30	1.10 $\pm$ 0.29
10.5 ..	3	..	..	..	2.93 $\pm$ 0.26	2.41 $\pm$ 0.22	2.28 $\pm$ 0.21	2.27 $\pm$ 0.20
9.5 ..	1	..	..	..				
8.5 ..	3	..	..	..				
7.5 ..	0	1	1	1				
6.5 ..	9	1	0	0				
5.5 ..	9	6	0	0				
4.5 ..	13	6	11	5				
3.5 ..	10	8	10	7				
2.5 ..	3	11	9	7				
1.5 ..	4	4	7	12				
0.5 ..	1	10	5	8				
<i>Lb. lost</i>								
-	3	7	3	9				
0.5 ..	2	2	3	8				
1.5 ..	..	2	0	0				
2.5 ..	..	1	..	2				
3.5 ..	..	..	..	1				
4.5 ..	..	..	..	1				
TOTALS ..	63	59	58	60				
					Biserial $r$ between M and N-M in respect of alteration in weight 0.5530 $\pm$ 0.09			
					0.1971 $\pm$ 0.09			

TABLE D.  
Frequency distribution of alteration in height during the experiment (hostel 1).

Alteration in height.	FIRST EXPERIMENT.		SECOND EXPERIMENT.	FIRST EXPERIMENT.		SECOND EXPERIMENT.	
	Milk (M).	No milk (N-M).		M group (63).	N-M group (59).	M group (58).	N-M group (60).
<i>Inches gained</i>							
+ 1.75 ..	..	..	Mean with standard error	0.61 $\pm$ 0.04	0.35 $\pm$ 0.04	0.69 $\pm$ 0.05	0.43 $\pm$ 0.04
1.50 ..	..	..	S. D. with standard error	0.31 $\pm$ 0.03	0.32 $\pm$ 0.03	0.40 $\pm$ 0.04	0.33 $\pm$ 0.03
1.25 ..	3	1	<i>Test for goodness of fit—</i>				
1.00 ..	7	5	(n) Degrees of freedom		6		7
0.75 ..	25	4	$\chi^2$		27.02		17.46
0.50 ..	13	11	P		Less than 0.01		0.02
0.25 ..	10	24	Significance		Significant.		Significant.
0.00 ..	5	14	<i>Correlation with alteration in weight—</i>				
..	..	..	r	0.2197 $\pm$ 0.13	0.3279 $\pm$ 0.13	0.20 $\pm$ 0.13	0.28 $\pm$ 0.13
			$\eta$	0.2921	0.4287	0.3026	0.3389
			Variance	Not significant.	Not significant.	Not significant.	Not significant.
			Regression	Linear.	Linear.	Linear.	Linear.
TOTALS...	63	59					

*Soya bean experiment (hostel 4).*

In respect of weight and height at the commencement of the periods of observation the two groups of girls (a) receiving soya bean (S) and (b) not receiving soya bean (N-S) are not distinguishable. The statistical constants are as follows:—

	Group S.	Group N-S.
Number under observation	38	37
(I) Mean weight (lb.) with standard error.	66.58 $\pm$ 3.04	65.51 $\pm$ 3.37
S. D. with standard error (in lb.).	18.71 $\pm$ 2.15	20.26 $\pm$ 2.35
(II) Mean height (inches) with standard error.	53.00 $\pm$ 0.67	52.59 $\pm$ 0.87
S. D. with standard error (height in inches).	4.12 $\pm$ 0.47	4.96 $\pm$ 0.58

The mean age was about 13 years. Height and weight means are slightly below those in hostel 1 (boys) but the mean age is almost the same.

Height increments during the period of observation were distributed as follows:—

Height increased by	1.25 inches.	1.00 inch.	0.75 inch.	0.50 inch.	0.25 inch.	0.00 inch.	TOTAL.
NUMBER OF GIRLS IN :							
Group S .. ..	..	1	9	15	9	4	38
Group N-S .. ..	1	1	6	14	12	3	37

The statistical constants obtained from these distributions are as under, and they show that the two groups did not experience differences in alteration of height:—

	Group S.	Group N-S.
Mean (in inches) ..	0.460 $\pm$ 0.04	0.453 $\pm$ 0.04
S. D. .. ..	0.247 $\pm$ 0.028	0.266 $\pm$ 0.031

In marked contrast with the general similarity of initial weight and height, and increase in height, in the two groups, there are differences as regards alteration in weight in favour of the group not receiving soya bean. In the S group eight

out of 38 cases increased in weight; in the N-S group, 18 out of 37. The latter group included four cases showing increases about 3 lb., which was the largest increase recorded in group S. In general, however, both groups showed *diminution* of weight. The distribution of weight increments, and the statistical constants derived therefrom are given in Table E.

TABLE E.

*Distribution of weight increments in S and N-S groups.*

Alteration in weight (lb.) at the end of the period of observation.	Group S receiving soya bean.	Group N-S not receiving soya bean.
<i>lb. gained</i>		
+		
8	..	1*
7	..	..
6	..	..
5	..	1
4	..	3
3	2	4
2	1	3
1	5	6
<i>Steady</i>		
..	4	4
<i>lb. lost</i>		
-		
1	5	2
2	5	2
3	3	2
4	2	2
5	3	1
6	3	3
7	4	..
8	..	..
9	..	2†
10	1*	..
11	..	1*
TOTALS .	38	37

Diminution (—) in weight (lb.)	S.	N-S.
Mean with standard error ..	2.37 ± 0.51	0.62 ± 0.69
S. D.    „    „    „    ..	3.150 ± 0.361	4.105 ± 0.488

The difference in the mean values as given above ( $1.75 \pm 0.86$ ) shows a greater decline in weight in the S group. Having regard to the standard error, this difference is significant on about a five per cent level. The distributions given in Table E include, however, one case in group S and four in the group N-S (marked with asterisks) which show somewhat abnormal variations. It may be feared that these large deviations might contribute to the significance in the differences

recorded above. Excluding the 'abnormal' cases, there remain 37 cases in group S and 33 in group N-S. The statistical constants yield by these are:—

Diminution (—) in weight.	Group S (modified).	Group N-S (modified).
Mean with standard error ..	2.16 $\pm$ 0.48	0.06 $\pm$ 0.54
S. D.    "       "       "    ..	2.926 $\pm$ 0.310	3.150 $\pm$ 0.361

The difference in the means is now  $2.10 \pm 0.72$ . This indicates that, at a two per cent level, the observed decrease in weight in the group receiving soya bean was significantly greater than the decrease in weight in the group of equal size not receiving soya bean.

There is thus a strong presumption, on statistical considerations at any rate, that the S group (which was not differentiated from the N-S group in respect of age, initial weight, initial height, or even in alteration in height at the end of the experiment), fared worse than the N-S group in respect of alteration in weight only.



# METABOLISM OF AMINO ACIDS IN HEART AND LUNG TISSUES.

BY

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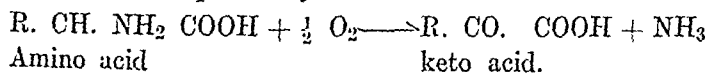
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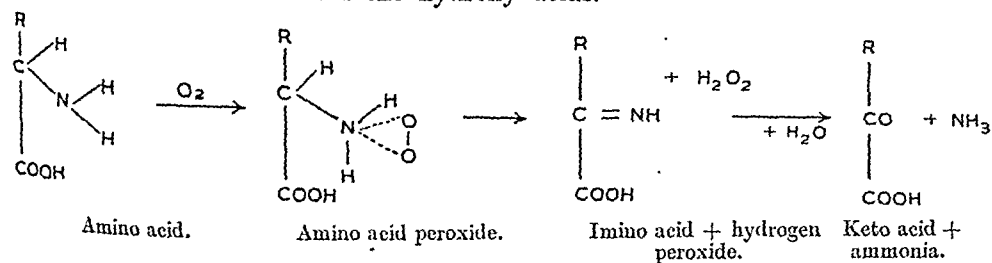
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NEUBAUER (1909, 1928) observed that on feeding phenyl-substituted amino acids foreign to the body, e.g., phenyl-glycine, the corresponding keto acid was excreted in urine. He thus showed that the deamination of l-amino acids in the mammalian body is accompanied by the oxidation of the  $\alpha$  c-atom



These results were confirmed by Knoop (1925, 1931) who showed that the reverse change, viz., the synthesis of amino acids *in vivo* and *in vitro* proceeded from the keto and not from the hydroxy acids.



Wieland and Bergel (1924) studied the behaviour of methylated amino acids in model systems and postulated that the primary step in deamination was dehydrogenation, removal of two hydrogen atoms, and the formation of the unsaturated imino acid. This preliminary formation of imino acid was confirmed by Knoop and Oesterlin (1925, 1927). Further work of Bergel and Bolz (1933, 1934) on the formation of a peroxide in the oxidation of methylated amino acids in model

systems and the work of Keilin and Hartree (1936) on coupled peroxidative oxidation suggest the following intermediate steps in the deamination of amino acids.

As regards the site of this deamination experiments suggested that it was localized in the liver. Bornstein's (1929) work furnishes many proofs of this theory.

Krebs (1933) has studied the deamination of amino acids by living tissue slices of different organs (excepting heart and lungs) of different animals. He found that the deamination proceeded in the liver and in the kidneys, the kidney cortex being the most active deaminating tissue per unit of weight (Krebs, 1935). The activity of liver was found to be about a third of that of kidney, but since the total amount of liver tissue in the body is much greater than the amount of kidney cortex, the function of the liver in deamination is considerable. Krebs also isolated the keto acids resulting from oxidative deamination as dinitro-phenyl hydrazones. The molecular ratio  $O_2 : NH_3 : \text{keto acid}$  was found to be 1 : 2 : 2. According to Krebs (1935) tissues of liver and kidney contain two different enzyme systems responsible for the oxidation of amino acids. The first is termed l-amino-acid deaminase, which oxidizes the natural amino acids. This cannot be extracted from cells, is inhibited by KCN, octyl alcohol and is destroyed by drying. The second system is termed d-amino-acid deaminase which oxidizes only the optical isomers of l-amino acids and these d-amino acids are foreign to the body. This system can be extracted from cells and is not inhibited by KCN, octyl alcohol or on drying.

Bernheim and Bernheim (1934) and also Bernheim (1935) have obtained results similar to those of Krebs.

While the metabolism of amino acids in the majority of vertebrate tissues like brain, retina, spleen, testicle, placenta, chorion, red blood cells, pancreas, salivary glands, and tumours, has been studied (Krebs, 1935), no attention appears to have been bestowed on heart and lung tissues. In this investigation the behaviour of some amino acids (mentioned later) in the heart and lung tissues has been studied.

#### EXPERIMENTAL PROCEDURE.

The reactions were carried out in a Barcroft-Warburg vessel with open manometers at 37°C. The rate of reaction was followed by determining the rate of oxygen uptake and the amount of ammonia was determined at the end of reaction. The inner cup of the reaction vessel contained 0.3 c.c. N NaOH and filter-paper as recommended by Dixon and Elliot (1930). Brodie's solution was used as the manometer fluid. The oxygen uptake was calculated from manometer readings. Ammonia was estimated by the method of Parnas *et al.* (1924, 1926).

Heart and lung tissues of rats were employed for the experiments as sources of enzymes. The organs were removed immediately after killing the animals by a blow and washed with Ringer's solution. In the case of heart tissues, the tissue-slice technique recommended by Warburg (1926) was employed to obtain thin slices. As the heart tissues are not sufficiently firm, slices thinner than 0.3 mm. could not be cut. In the case of lung tissues slices were cut thin by scissors.

In reaction vessels the tissues were suspended in phosphate saline. The amino acids were neutralized before being added. The gas space was filled with oxygen. The volume of the reaction vessels was about 30 c.c. to 35 c.c. The



volume of the reaction mixture was always 5 c.c. The final concentration of amino acids was  $\frac{M}{50}$ . About 100 mg. of tissue (i.e., 15 mg. to 20 mg. of dry weight) were taken. The amount of ammonia formed is expressed in cubic millimetres,  $\mu$ l (17 mg.  $\text{NH}_3 = 22,400 \mu$ l), in order that it might be directly compared with the volume of oxygen consumed.

The following amino acids were investigated :—

1. l-cystine.
2. l-proline.
3. d-l-serine.
4. d-arginine.
5. l-histidine.
6. l-tyrosine.
7. d-glutamic acid.
8. d-l-methionine.
9. d-l-alanine.
10. glycine.
11. glycylglycine.
12. d-l-phenylalanine.
13. l-leucine.

Of these different amino acids only the l-cystine and l-proline were deaminized by enzymes of heart tissue and only l-cystine was deaminized by lung tissues, the rest of the amino acids being unaffected. Even cystine and proline were deaminized very slowly and  $Q_{O_2}$  and  $Q_{\text{NH}_3}$  represent mean value of oxygen absorption and ammonia formation for 30 mg. tissue per hour. The results obtained are given in Table I (heart) and Table II (lung). The blank experiments with tissues alone in phosphate saline were carried out and taken into consideration in finding out  $Q_{O_2}$  and  $Q_{\text{NH}_3}$ .

TABLE I.

*Oxidation by heart tissue.*

Substance.	$Q_{O_2}$	$Q_{\text{NH}_3}$	$\frac{Q_{O_2}}{Q_{\text{NH}_3}}$
l-cystine .. ..	15.28	25.77	$\frac{1}{1.69}$
l-cystine and KCN ..	16.52	31.21	$\frac{1}{1.87}$
l-cystine and octyl alcohol ..	12.20	21.70	$\frac{1}{1.94}$
l-proline .. ..	76.54	107.64	$\frac{1}{1.40}$
Proline and octyl alcohol ..	45.72	38.96	..
Proline and KCN .. ..	6.16	44.15	..

TABLE II.  
*Oxidation by lung tissue.*

Substance.	$Q_{O_2}$	$Q_{NH_3}$	$\frac{Q_{O_2}}{Q_{NH_3}}$
l-cystine .. ..	21.40	40.50	$\frac{1}{1.90}$
Cystine and KCN .. ..	22.24	45.00	$\frac{1}{2.02}$
Cystine and octyl alcohol ..	17.91	32.37	$\frac{1}{1.80}$

The rate of oxygen uptake with cystine and proline in heart and with cystine in lung tissues is indicated in Tables III and IV and represented in Graphs 1, 2, and 3:—

TABLE III.  
*Oxidation by heart tissue.*

Substance.	OXYGEN ABSORPTION IN $\mu$ l.						
	Time in minutes.						
	0	30	60	90	120	150	180
Cystine .. ..	0	2.37	4.67	7.30	9.34	11.29	13.25
Cystine and KCN .. ..	0	3.85	7.52	10.8	13.2	15.2	16.3
Cystine and octyl alcohol ..	0	4.43	7.76	9.8	11.09	12.2	12.94
l-proline .. ..	0	4.41	10.9	20	35.8	54	78.03
l-proline and octyl alcohol ..	0	4.05	7.85	16.7	22.77	32.28	46.85
l-proline and KCN .. ..	0	0.29	0.73	1.7	2.66	4.3	6.36

TABLE IV.

*Rate of oxidation by lung tissue.*

Substance.	OXYGEN ABSORPTION IN $\mu$ l.						
	Time in minutes.						
	0	30	60	90	120	150	180
l-cystine .. ..	0	3.7	6.9	8.9	11.16	13.83	16.2
Cystine and KCN .. ..	0	3.9	7.9	11.2	14.02	15.91	17.0
l-cystine and octyl alcohol ..	0	6	10.9	15.4	..	16.4	17.91

It will be seen that the ratio of  $\frac{Q_{O_2}}{Q_{NH_3}}$  is very seldom equal to the theoretical ratio of 1/2. This is undoubtedly due to other reactions in which oxygen is absorbed, interfering. The best approximation for the rate of deamination is obtained by measuring the rate of formation of ammonia, because ammonia formation and consumption by other reactions are generally small.

As already mentioned in the introduction, Krebs (1935) postulates the existence of two enzymes: l-amino-acid deaminase and d-amino-acid deaminase, of which the latter is said to be unaffected by KCN, octyl alcohol, or by drying, while the former like the respiration of cells, is inhibited by KCN and octyl alcohol. Experiments were, therefore, carried out in presence of KCN and octyl alcohol, and the results obtained are already indicated in Tables III and IV.

It will be seen that the deaminations of l-cystine, both in heart and lung tissues, is scarcely affected either by KCN or by octyl alcohol. This is contrary to the fundamental postulate of Krebs, who maintains that l-amino-acid deaminase like the respiration of cells is inhibited by KCN and by octyl alcohol. In the case of l-proline, in which the oxidative breakdown is very complicated as shown by Bernheim (*loc. cit.*) and by Krebs (1935), the deamination is almost completely inhibited by KCN. Octyl alcohol also has got an inhibitory effect but this is not so pronounced, as in the case of KCN.

In view of this observation, therefore, it is doubtful whether the characteristics of the two deaminases as defined by Krebs are wholly true.

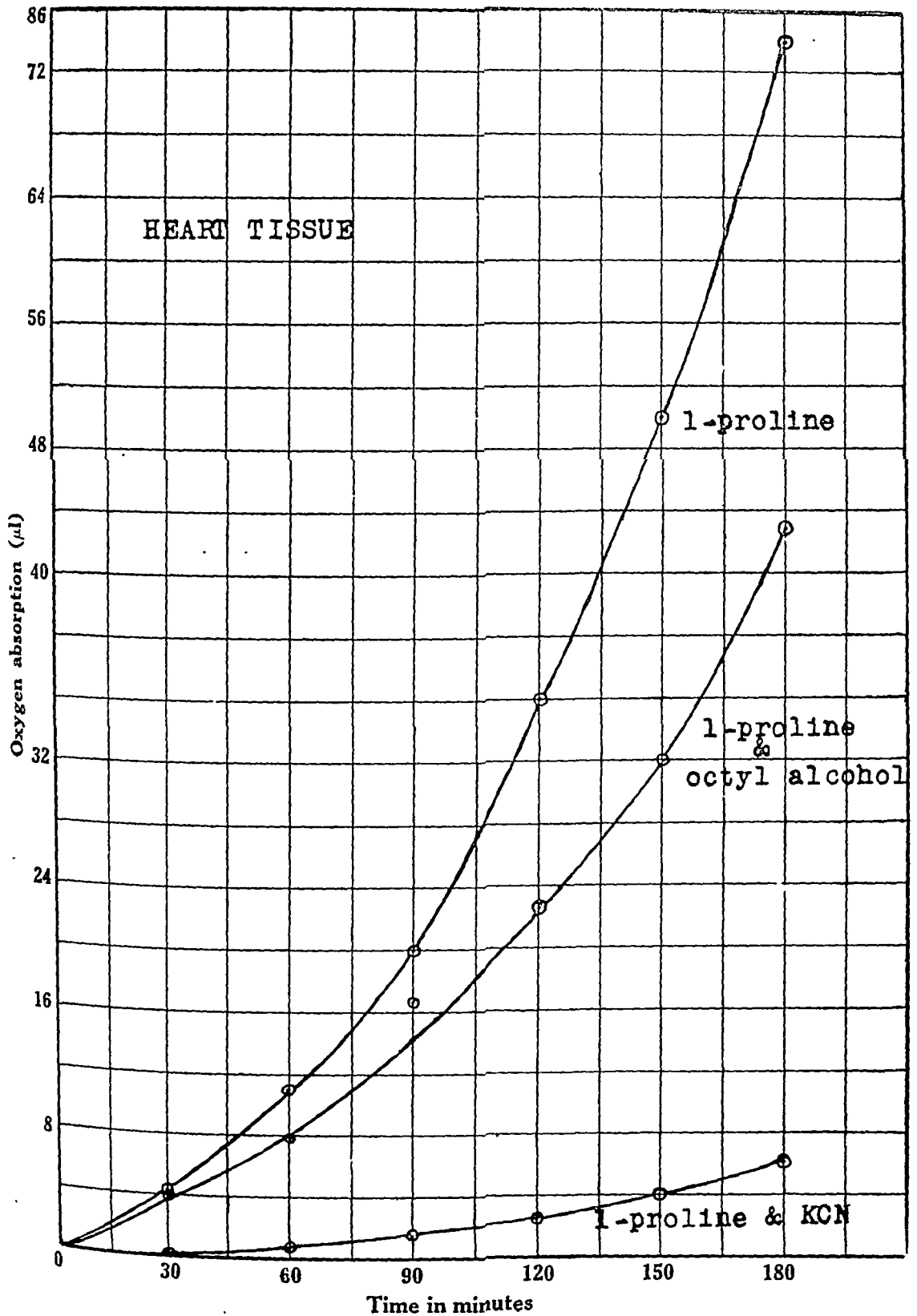
Graph 1 is a line graph showing the amount of oxygen absorbed (in  $\mu\text{l}$ ) by heart tissue over time (in minutes) under different conditions. The y-axis represents 'Oxygen absorbed ( $\mu\text{l}$ )' and ranges from 0 to 32 in increments of 8. The x-axis represents 'Time in minutes' and ranges from 0 to 180 in increments of 30. The graph shows four distinct curves, all starting at (0,0) and increasing over time. The curves are labeled as follows: 'Heart tissue' (the highest curve), 'l-cystine KCN' (the second highest curve), 'l-cystine' (the third highest curve), and 'octyl alcohol' (the lowest curve). The 'l-cystine' curve is also labeled with an arrow pointing to it from the right side of the graph.

Time in minutes	Heart tissue ( $\mu\text{l}$ )	l-cystine KCN ( $\mu\text{l}$ )	l-cystine ( $\mu\text{l}$ )	octyl alcohol ( $\mu\text{l}$ )
0	0	0	0	0
30	6	4	3	2
60	10	7	5	4
90	14	10	8	6
120	16	12	10	8
150	17	14	12	10
180	18	15	13	11

The graph illustrates the rate of oxygen absorption by lung tissue over an 180-minute period. The y-axis represents the volume of oxygen absorbed in microliters (μl), ranging from 0 to 32. The x-axis represents time in minutes, ranging from 0 to 180. Three experimental conditions are plotted: 1-cystine (open circles), 1-cystine & KCl (open circles), and 1-cystine & octyl alcohol (open circles). The 1-cystine & KCl condition shows the highest oxygen absorption, followed by 1-cystine & octyl alcohol, and then 1-cystine alone.

Time in minutes	1-cystine (μl)	1-cystine & KCl (μl)	1-cystine & octyl alcohol (μl)
0	0	0	0
30	2.5	4.5	3.5
60	4.5	7.5	6.5
90	6.5	10.5	9.5
120	8.5	13.5	12.5
150	10.5	15.5	14.5
180	12.5	16.5	15.5

GRAPH 3.



## SUMMARY.

Of the different amino acids investigated, viz., l-cystine, l-proline, d-l-serine, d-arginine, l-histidine, l-tryptophane, d-glutamic acid, d-l-methionine, d-l-alanine, glycine, glycylglycine, d-l-phenylalanine, and l-leucine, only l-cystine and l-proline were found to undergo oxidative deamination in heart and l-cystine alone in lung tissues of rats. The oxygen absorption was measured by Barcroft-Warburg respirometers and ammonia was estimated by the method of Parnas. The deamination of l-cystine both in heart and lung tissues is not affected by KCN or octyl alcohol. The oxidative deamination of l-proline by heart tissues is completely inhibited by KCN and partially by octyl alcohol.

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## THE ACTION OF AJMALINE ON NERVE IMPULSES.

BY

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### INTRODUCTION.

CONSIDERABLE attention has been paid during recent years to *Rauwolfia serpentina*, a plant belonging to N. O. *Apocyanaceæ*. The root of this plant is largely used in the Hindu medicine and remarkable qualities are attributed to it with regard to its effects in various nervous diseases. In certain parts of the United Provinces and Bihar it is largely used as a remedy for insomnia and insanity. Sen and Bose (1931) carried out a chemical examination of the roots and reported the presence of two alkaloids. A decoction of the root proved effective in patients suffering from insomnia, high blood pressure, and certain types of hyper-excitability of the central nervous system. Siddique and Siddique (1931) investigated the chemistry of this root more in detail and found a number of alkaloids which they divided into two groups: the *ajmaline* group and the *serpentine* group. Chopra, Gupta and Mukherjee (1933) examined the roots, and found an alkaloid very similar to the *ajmaline* of Siddique and Siddique but differing slightly in its melting point. An extensive pharmacological and toxicological investigation on this alkaloid showed that it possessed marked depressant properties on the central nervous system and that it could be tolerated in fairly large doses. There was also evidence that there was definite inhibition of the activity of the nerve cells in the body.

The present research was undertaken to investigate, in more detail, the nature of the depressant effects produced on the nervous structures of the body by the alkaloid *ajmaline*, which is one of the most important alkaloids contained in the root.

## TECHNIQUE.

A nerve-muscle preparation was selected from the middle toe muscle of a frog (*Bufo melanostictus*) in connection with the peroneal nerve which is fairly long and continuous with the sciatic. This was placed inside a double chambered vessel of ebonite which was separated into two compartments by means of a celluloid partition provided with a small slit through which the nerve fibre could pass. The preparation was so placed that the muscle was kept in one chamber, while the nerve would remain in the other. In order to generate proprioceptive impulses a device similar to the 'dash-pot' arrangement of Matthews (1929) was employed. A thread was attached to a loading pulley on which hung a weight. The weight was lowered at a constant speed by some mechanical device so that when the weight falls it draws up the piston of a glass syringe which is made to suck up some fluid and thus does not allow the tension to act suddenly on the muscle. The muscle after separation was fixed to one of the chambers which had been partly filled with the frog saline (Ringer's). The nerve fibre was then led through the slit into the next chamber and two fine-pointed silver-silver chloride electrodes, prepared according to the method of Noyes and Ellis (1917), were made to touch two points on it. The whole arrangement was then introduced into a chamber which was kept uniformly moist, so that the humidity and temperature would remain constant during the period of the experiment. The nerve was so arranged as not to come in contact with the fluid bathing the muscle. Connections from the non-polarizable silver-silver chloride electrodes were led to the amplifier of an oscillograph devised by Chopra and Das (1935) and the nerve impulses generated were recorded photographically.

The soluble hydrochloride of ajmaline in concentrations ranging from 1 in 200,000 and upwards was used in these experiments. The hydrochloride was used in the Ringer's solution. After taking the record for the normal control (Ringer's solution) the alkaloid solutions of definite concentrations were used in the muscle compartment and separate records taken for each solution after the muscle had been bathed therein for about 10 minutes and action become normal in every way. It was not possible to study more than a few different concentrations with the same nerve-muscle preparations. The records for concentrations of 1 in 200,000, 1 in 100,000, and 1 in 50,000 are given in the Graph, fig. 2 (A, B, C and D).

According to Adrian and Zotterman (1926) the frequency of proprioceptive impulses is known to change with the amount of tension to which the muscle is subjected. This was confirmed in our own experiments and hence a constant weight of 4 grammes was used to stretch the muscle throughout the experiment.

It was observed in the course of this work that at the beginning and immediately after the muscle had been stretched the frequency and amplitude were high, but they gradually came down to a constant level in a very short time when the impulses were of a more or less constant frequency and amplitude persisted for an indefinite time (*vide* Graph, fig. 1, a). Similar observations have been recorded by Adrian (1932) in the case of sensory discharge from vagal endings in the lungs, where the frequency of the discharge has been found to fall appreciably in the first second from 150 to 115 per second. Bogue and Rosenberg (1936) have also recorded in a recent paper that monophasic wave generated by a



# GRAPH.

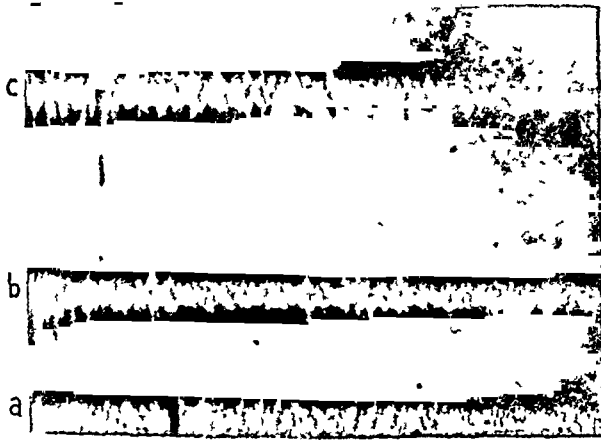


Fig. 1.—Showing the initial maximum of frequency  
(a) Discharge from a normal nerve, i.e., without treatment with ajmaline. (b) Nerve treated with 1 in 100,000 ajmaline hydrochloride solution (c) Nerve treated with 1 in 50,000 ajmaline hydrochloride solution.

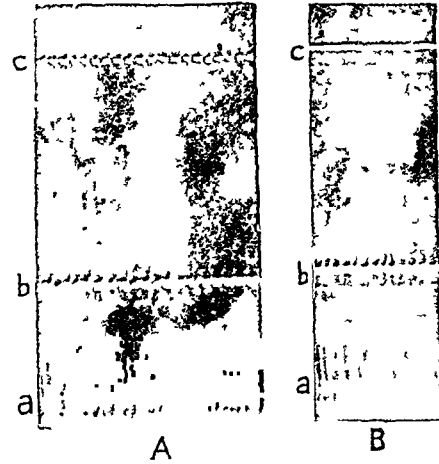


Fig. 3.—Showing the effect of different concentrations of ajmaline on the transected nerve A—(a) Normal control, (b) 1 in 100,000 ajmaline, and (c) 1 in 50,000 ajmaline B—(a) Normal control, (b) 1 in 100,000 ajmaline, and (c) 1 in 100 ajmaline.

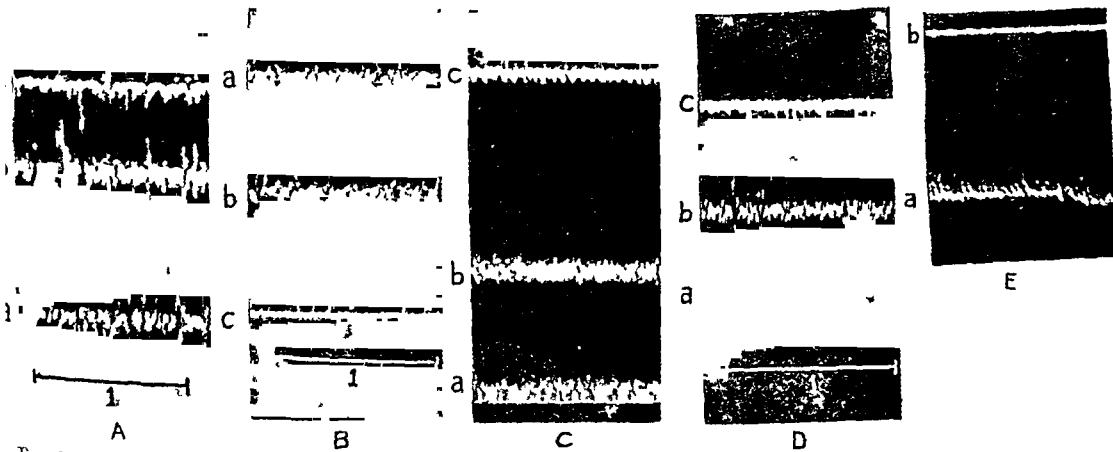


Fig. 2.—Showing the effect of different concentrations of ajmaline solution on the whole nerve A—(a) Normal control, (b) 1 in 200,000 ajmaline solution, and (c) 1 in 100,000 ajmaline solution B, C and D—(a) Normal control, (b) 1 in 100,000 ajmaline solution, and (c) 1 in 50,000 ajmaline solution. E—(a) Normal control, and (b) 1 in 100 ajmaline hydrochloride solution.



single electrical stimulation of nerves rises initially to a peak value of 10 to 15 millivolts in their experiments with sensory bundles which gradually diminishes to a lower and more stationary level. All our records, therefore, were taken after the frequency and amplitude had become uniform. An interval of 12 to 15 minutes was allowed between two consecutive stimulations of the muscle so that the effect of the previous stimulus might die out completely before a second one was imparted, and the muscle was not fatigued in any way. The preparations in general would keep for 3 to 4 hours during which time the experiments were to be finished.

### RESULTS.

A study of records in the Graph, fig. 2, shows that in concentration of 1 in 200,000 the frequency is definitely increased. Preparations from different frogs were studied for each concentration in the way mentioned above. In some instances it was observed that at the concentration of 1 in 200,000 there was no enhancement of impulse frequency, but in no case was there decrease below the normal control (records not given here). At concentration of 1 in 100,000 there was a lowering of frequency and amplitude in the majority of preparations studied as compared with the normal control; in a few cases there was no change at all. At still higher concentrations, however, the impulses uniformly slowed down till at last at a certain concentration, viz., with 1 in 100 in one case, the impulses were found to be completely stopped (Graph, fig. 2, E).

In this connection it was also observed that the initial maximum frequency was less prominent as the concentration was increased. In other words, the maximum of frequency that could be observed immediately after the application of stimulus showed a diminution of both frequency and duration as the concentration was increased (Graph, fig. 1, *b* and *c*). These observations are in conformity with those of Bronk (1929) with strychnine hydrochloride.

In the foregoing records the whole nerve was stimulated. The curves obtained represent the resultant effect of stimulation of the individual fibres and as such they are very complex. It was, therefore, attempted to study the effect of stimulation of transected nerve so that only a very small number of fibres was subjected to stimulation. The transection was done with the help of a dissecting microscope and glass pointers according to the method described by Adrian and Bronk (1928) in such a way as to cause least disturbance to the fibres. The dissection was carried on till few fibres necessary for conduction of impulses were left intact. The records are given in the Graph, fig. 3, A and B. It will be seen that the curves in these cases are simpler and the changes of frequency can be easily pursued.

### INTERPRETATION.

From the records given it is obvious that at higher concentrations the alkaloid diminishes the frequency of nerve impulses so much so that at certain concentrations transmission is practically stopped. In some of the cases of lower concentrations, viz., at 1 in 200,000, the frequency had been found to increase. This observation is very interesting in view of the fact that Chopra, Gupta and Mukherjee (*loc. cit.*) found that the first effect of small doses of the alkaloid was stimulation.

The absence of any effect on the frequency, as seen in many cases at a concentration of 1 in 200,000 and sometimes with 1 in 100,000, may be explained in the following way: According to Adrian and Zotterman (*loc. cit.*) the frequency of proprioceptive impulses coming from end-organs of nerves is known to increase with the stimulus. The rise in frequency becomes less and less as the stimulus increases and finally reaches a maximum after which there can be no increase of frequency with further increase of stimulus. It is, therefore, quite likely that the load used by us was able in some preparations to bring about a maximum discharging rate and any further sensitization brought about by the alkaloid in dilute solutions was marked by the effect of the tension.

It is, however, clear that at higher concentrations of the alkaloid the frequency of discharge of sensory impulses from stretched muscle is diminished. The question next comes as to how this diminution is caused? Are the end-organs desensitized or is the conductivity of the sensory fibres diminished? Although the nerve fibres were kept isolated from the muscle and no alkaloidal solution could go directly to the nerve chamber, the possibility that some of the alkaloid, by diffusion through the muscle, acted on the intramuscular sensory fibres remained.

If the nerve fibres were directly exposed to the action of the alkaloid, no change in frequency could be observed as compared to normal controls arranged similarly with Ringer's solution only. In addition to this the study of discharge from transected nerve which gives the discharge from only a very few end-organs, if not a single end-organ, tends to show that probably the diminution of the activity of the end-organs is responsible for the effect produced on the discharge frequency by the alkaloid.

#### SUMMARY AND CONCLUSION.

The alkaloid ajmaline extracted from the roots of *Rauwolfia serpentina* diminishes the discharge frequency of nerve impulses. This inhibitory action on the nerve fibres becomes more prominent as the concentration of the alkaloid increases until at a certain maximal concentration no impulse passes through them.

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## ON THE ACTIONS OF NEO-STIBOSAN, UREA-STIBAMINE, AND HISTAMINE ON FROG'S HEART.

BY

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THE actions of neo-stibosan, urea-stibamine, and histamine have been studied on the heart of frogs by perfusion with Ringer's fluid\* containing the drugs in suitable proportions. To avoid certain drawbacks attendant on the ordinary methods of perfusion, a special method of perfusion was devised where the pressure of the perfusing fluid could be maintained at a fairly constant level and the drug to be tested could be allowed to remain in contact with the cardiac tissue for prolonged periods.

The apparatus used for perfusion, as shown in the Diagram, consists of two cylinders, A and B, of uniform bore and graduation and provided with a stop-cock. A contains Ringer's saline and B contains the solution of the drug in Ringer's saline. A and B are connected by rubber tubes C and D to the perfusion cannula F. A T-tube is inserted in the course of each of two tubes C and D, not shown in the Diagram, for draining off the solutions from A and B. A is fastened to the stand M and B to a graduated stand N by the clamp O which is fixed to a spiral P inserted within the stand N. The spiral can be moved up and down by the lever Q. The level of liquid in B is indicated by a signal B' attached to a rod fixed to the stand N. In consequence of this arrangement the cylinder B can be moved up and down so that the level of liquid contained therein may remain stationary at the point indicated by the signal B'. The rubber tube D leading out from B is connected to a glass tube provided with a glass stop-cock, the other end of the glass tube being connected by a rubber tube to the cannula F. A circular brass disc with graduations at the rim is fitted to the stop-cock E at each edge of which is attached a brass piece pointing to the scale on the brass disc. When the stop-cock is wide open, the brass piece at one end points to zero. When the stop-cock is moved on either side of zero, the outflow of liquid through the glass tube is restricted. If the level of liquid in B is kept at a constant

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\* Bayliss' (1924) formula for Ringer's solution for the heart of frog was adopted.

height and if the pointer attached to the stop-cock is fixed to a particular graduation on the scale mentioned above, then the liquid flowing out of the perfusion cannula kept at a constant position will always be and is also found to be the same. The frog is placed on a Palmer's crank myograph with a bigger cork-board than usual and a screw arrangement at the base for turning the myograph slowly from side to side. The T-piece carrying the myograph is fixed to a device R, which enables the myograph lever to be placed tangentially against the drum by rotation of the myograph.

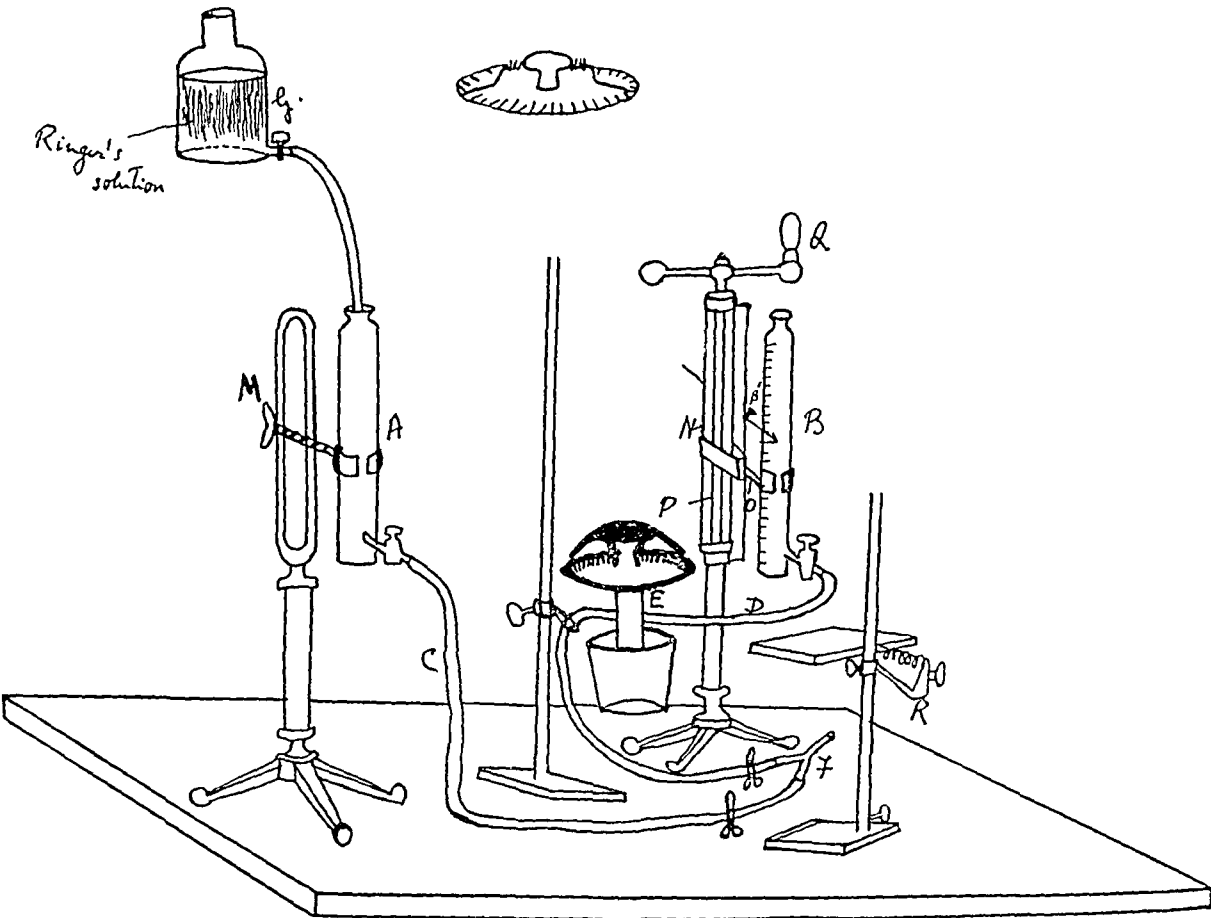


DIAGRAM.

*Modus operandi.*

A and B are filled with Ringer's solution. Their stop-cocks are then opened out so that the tubes C and D up to the cannula F are filled with the solution without any air-bubbles being left in them. Now the height of the level of liquid in A is noted and the signal B' attached to B is fixed at the same height as the level of liquid in A. The level of liquid in B is then adjusted to the height of the pointer B' by turning in or out the lever Q. Now the outflow of liquid from A with its stop-cock fully open is measured carefully for a few seconds. The outflow of liquid

from B is then measured for the same period under identical conditions. If there is any difference in the rate of outflow, and if the rate of outflow from B is greater, the rate can be regulated by the stop-cock E and brought on a line with that of A and vice versa.

After the apparatus has been thus adjusted, a pithed frog with its heart exposed is placed on the myograph. One branch of its aorta is then ligatured and an incision is made in the other branch. A small opening is then made in the inferior *vena cava* as far away from the heart as is possible. When these incisions are made the heart may be freed from blood quickly. After washing off the blood with Ringer's solution the cannula is thrust into the small opening and a fine glass tube is inserted into the incision in the aorta. The height of the cannula is then noted and the levels of liquid in A and B are then placed at a height greater than that of the cannula by 1" to 1½", for it has been found that the heart of frogs works best at a fluid pressure of 1" to 1½". The stop-cock of A is then opened out and Ringer's solution is allowed to perfuse through the heart. Ringer's solution in both A and B is aerated from time to time, when it is being circulated through the heart, through a glass tube connected by a rubber tube with a mouth-piece. The level of liquid in A is kept constant by connecting G, which contains Ringer's solution, with A and adjusting the outflow from G with the outflow from A. The level of liquid in B is kept constant by the method mentioned above. When the Ringer's solution circulates through the heart, it begins to beat and within a short time the beats become normal and tracings are recorded. Now B is opened out and A shut off immediately. The features of the heart-beat are not usually changed by shunting off the outflow from A to B. But if any change is noticed, the normal features are restored within a short time provided the rate of outflow from B is kept constant. After these adjustments are made, the drug solution is added to B and the heart is perfused with this diluted drug solution for a period of time and the tracings are taken.

### RESULTS.

(a) *Neo-stibosan*.—The effects of neo-stibosan in varying concentrations, viz., 4.5 mg. per cent, 7 mg. per cent, 9 mg. per cent, and 13 mg. per cent solutions, on frog's heart were studied. They were found to be similar and it was observed that neither during the course of perfusion of the heart with the drug solution, nor afterwards, i.e., during the washing away of the drug with Ringer's solution, any changes in the heart-beat were produced.

If 0.05 g. be taken as the initial dose for human beings, then the approximate dose for a frog weighing 100 grammes is found to be about 0.05 g. ÷ 100. Now, as the total volume of blood in a frog weighing 100 g. is found to be nearly 12 c.c., the percentage in concentration of neo-stibosan solution in the case of frogs comparable to the human dose is  $\frac{0.05}{100} \times 8.4$ , nearly, or  $0.0005 \times 8.4$  grammes per cent, i.e., 0.0042 gramme per cent, i.e., 4.2 mg. per cent.

(b) *Urea-stibamine*.—With 0.25 mg. per cent of urea-stibamine solution no effects on the heart were noticed. With 0.5 mg. per cent of the solution a slight augmentation of the beat was noticed. With 1 mg. per cent of the solution a little

more augmentation of the beat but with a slowed frequency was observed. With 3.8 mg. per cent of the solution there was marked augmentation after 7 minutes' perfusion with the drug. Although this augmentation effect was maintained up to 30 minutes, it was passing off gradually towards the latter part of this period. As soon as Ringer's fluid was introduced the heart-beats became almost immediately depressed. With 4.5 mg. per cent of the solution a slight augmentation of the beat was noticed at the beginning. A marked depression with slowing of the heart-beat was observed after perfusion for 2 minutes and 5 minutes after perfusion the ventricular beats disappeared. The ventricular beats reappeared after  $7\frac{1}{2}$  minutes and again disappeared after 9 minutes. A washing with Ringer's solution at this stage restored the normal beats after 3 minutes. After 5 minutes the beats became augmented and a further increase was marked after 6 minutes. The beats became smaller in amplitude, slower in rhythm at times, and irregular from an interval of 6 minutes to 21 minutes. Normal and regular beats were obtained after 22 minutes. The experiment was repeated and the same effects were obtained but more quickly than before.

(c) *Histamine*.—A perfusion with 0.05 mg. per cent solution did not produce any effect on the heart. With 0.12 mg. per cent of the solution a pronounced action was seen. There was an initial depression, followed after 3 minutes by irregular beats leading to complete stoppage of the heart in 5 minutes. A few ventricular beats were found to slip out at times within an interval of 10 minutes of the complete arrest of the heart. After 15 minutes, perfusion with Ringer's solution was resumed and normal beats were restored after 5 minutes. These effects were easily duplicated.

#### DISCUSSION.

The experiment with neo-stibosan solution demonstrates that the drug does not affect frog's heart at the dose corresponding to the initial dose for a human being, and also at double or treble that dose. With urea-stibamine, it is noticed that the drug is highly depressant to frog's heart at the concentration nearly corresponding to the initial human dose. At a dose lower than the initial human dose, viz., at 3.8 mg. per cent solution, the drug leads to gradual depression of the heart on prolonged perfusion. It is also seen that the drug has an augmentatory effect on the heart-beat at lower doses, viz., from 0.5 mg. per cent to 3.8 mg. per cent, but the more the augmentation, the greater is the risk of the heart being depressed on prolonged perfusion. A noteworthy feature in the experiment was the phase of marked augmentation of the heart-beat when the organ was washed with Ringer's fluid after it was depressed with 4.5 mg. per cent of the urea-stibamine solution. This effect might be produced in either of the following ways :—

- (a) As the heart was being washed with Ringer's solution after perfusion with urea-stibamine solution, the latter obviously attained lower concentrations at which the heart would be augmented. Accordingly augmentation took place during washing. The subsequent depression of the heart and irregularity of beats were probably due to the exaggerated activity of the affected heart.
- (b) This phase of augmentation probably indicated a release of the activity of the heart after a period of inhibition as soon as the cause of this



inhibition was removed. The subsequent depression and irregularity of the heart were the results of the persistence of the depressant effect of the drug even after it had been washed.

It was observed that there were several periods of depression alternating with phases of augmentation during washing with Ringer's solution and even long after such washing was started. It is thus obvious that these phases of augmentation were not caused by lower concentrations of urea-stibamine during washing but were probably brought about by the sudden release of activity of the heart as is mentioned in (b), the alternation between phases of augmentation and depression being probably due to a rivalry between the release of activity of the heart and the persistence of the depressant effect of the drug even after its withdrawal from the circulating fluid.

Histamine is a strong depressant of the frog's heart at a very low concentration of 0.12 mg. per cent which corresponds to a dose of 0.0144 mg. for injection (12 c.c. being taken as the volume of blood in a frog weighing, on an average, 100 grammes). The corresponding human dose for injection would be  $0.0144 \text{ mg.} \times 100$ , i.e., 1.44 mg., and at this dose the human heart is expected to be depressed within a short time although there may not be any action on the heart at the dose of 0.6 mg.

#### SUMMARY AND CONCLUSIONS.

A method of perfusion of frog's heart is described and special methods have been devised to keep the pressure of the perfusion fluid constant and the friction of the lever against the drum invariable.

The effects of perfusion of the frog's heart with the two well-known organic antimony compounds, e.g., urea-stibamine and neo-stibosan, at varying concentrations have been studied. It is concluded after comparing these effects that neo-stibosan is non-toxic to frog's heart, not only at the concentration corresponding to the initial human dose, but at double or treble that concentration; whereas urea-stibamine produces temporary augmentation followed by depression at the concentration slightly lower than the corresponding initial human dose, but at a slightly higher concentration it is highly toxic.

Histamine has no action on frog's heart at a concentration of 0.05 mg. per cent, but has a markedly depressant action on the heart when the concentration of the solution reaches 0.12 mg. per cent. The corresponding human dose for injection at which the human heart is expected to be depressed is 1.44 mg., although there may not be any action on the human heart at the dose of 0.6 mg.

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## SOME INORGANIC PREPARATIONS OF THE INDIAN INDIGENOUS MEDICINE.

### Part IV.

#### *RAUPYA BHASMA* (REDUCED SILVER).

BY

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THE use of silver in the indigenous system of medicine of India dates back to remote antiquity. Its Sanskrit name *Raupya* is found in many of the early works of Hindu medicine and there is ample evidence in the literature to show that silver held an important place in therapeutics. Its use was, however, small as compared with metals like iron and tin and the number of preparations was limited.

#### *Preparation of Raupya Bhasma (reduced silver).*

Like all other metallic preparations of Ayurvedic medicine, silver is also used in the form of crude compounds formed after subjecting it to complicated processes of so-called 'purification', which convert it to a fine greyish-black powder. The methods used for the 'purification' and reduction are numerous. Some of the more important ones are given below :—

(1) Silver is 'purified' for preparing its 'ash' by melting it with lead and borax. (2) Thin sheets of silver are heated to redness and steeped thrice in each of the following, viz., oil, whey, kanji, cow's urine, and extract of *Kulatha Kalai* (*Dolichos uniflorus*). The metal thus 'purified' is suitable for

entering into the composition of medicinal preparations. The sheets of purified silver are smeared with *Kajjali* which is prepared by mixing two parts of sulphur and one part of mercury ground with juice of *Jambira* (*Citrus acida*). It is then heated in *Gajaputa*. The product thus obtained is called *Raupya Bhasma* or reduced silver. (3) Silver leaves as purified above are cut into small pieces and powdered with an equal quantity of mercury. It is next pounded with juice of *Citrus medica* and subjected to the process of roasting known as *Putapaka*. By repeating the process thrice pure 'ashes' of silver may be obtained. (4) A paste is made by mixing powdered orpiment and another paste is made by mixing powdered pomegranate bark, acacia leaves and juice of aloe leaves (*Aloe indica*). These two pastes are thoroughly mixed and a bolus is made with it. In the centre of this, pure refined silver leaf is placed in the shape of a ball and the whole is covered with clay. It is then roasted and calcined. (5) Silver leaf is rubbed with mercury and the juice of *Atrocarpus lakoocha*. The resulting paste is then embedded in sulphur and heated in a covered crucible in a sand-bath. When cold, the mass is once more rubbed with orpiment and acid and roasted twelve times. By this process the silver is reduced to an ash-like substance. (6) Four parts of silver leaves are rubbed with one part of orpiment and lemon juice and the mixture is roasted. The process is repeated 14 times and thus the silver is completely reduced. (7) Silver leaves are mixed with twice the weight of cinnabar and heated in the subliming apparatus called the *Urdhapatan Jantra*. This process is repeated 14 times and the resulting compound is a fine greyish-black powder with minute shining white particles intermixed with it.

A sample of *Raupya Bhasma* obtained from the Kalpataru Ayurvedic Works, Calcutta, was subjected to analysis. In appearance it was a greyish-black amorphous powder with an admixture of very small white particles. Its chemical composition as the result of our qualitative and quantitative analyses is given below :—

	Per cent.
Silver metallic .. .. .	69·670
Sulphur .. .. .	14·806
Ferric oxide, $\text{Fe}_2\text{O}_3$ .. .. .	7·830
Alumina, $\text{Al}_2\text{O}_3$ .. .. .	2·250
Cupric oxide, $\text{CuO}$ .. .. .	0·890
Phosphate, $\text{P}_2\text{O}_5$ .. .. .	1·080
Silica, $\text{SiO}_2$ .. .. .	1·160
Lime, $\text{CaO}$ .. .. .	0·880
Potash, $\text{K}_2\text{O}$ .. .. .	0·141
Soda, $\text{Na}_2\text{O}$ .. .. .	0·054
Sulphuric anhydride, $\text{SO}_3$ .. .. .	0·935
Moisture and other constituents .. .. .	0·304
<hr/>	
TOTAL .. .. .	100·000

According to some authors '*Raupya Bhasma*' is an impure oxide of silver. The sample analysed by us proved to be more of the nature of a sulphide. This difference in composition may be due to the different methods of preparation used. The preparation according to the methods (2) and (3) in which little or no sulphur has been used give the oxide, whereas the other preparations would give the sulphide.

#### *Pharmacology and therapeutics.*

*Raupya Bhasma* being an insoluble inorganic compound, its pharmacological action is difficult to test. To test the popular belief regarding the therapeutic efficacy of *Raupya Bhasma* in nervous disorders, e.g., epilepsy, chorea, and neuritis, an attempt was made to see whether it has had any effect on peripheral nerves.

A nerve-muscle preparation of the gastrocnemius muscle of the frog was treated with a 3 per cent suspension of the drug in normal saline and the nerve-impulse changes were recorded by means of an oscillograph. Preliminary experiments showed that there was no change of impulse frequency when the muscle alone was exposed to *Raupya Bhasma* suspension as compared with the normal control. On the other hand, when the nerve fibres were bathed in the suspension the discharge frequency showed a slight diminution. Though these results cannot be considered definitely indicative, they are perhaps suggestive and indicate that the supposed action of silver on the nerves is not purely based on popular belief but may have some scientific basis.

In modern therapeutics the use of silver is not extensive. Silver preparations are generally used externally for removal of newly-formed tissues, especially in chronic inflammations and ulcers. The main principle of its therapeutic use consists in the capacity of silver to combine chemically with proteins to form proteinates. In silver nitrate, the concentration of silver-ions is high and the destruction of tissues is more drastic and hence it acts as a caustic. Where milder actions are required, silver proteinates, e.g., silver caseinates, or colloidal silver preparations, are used since they contain a much lower concentration of ions than electrolytic salts of silver.

The use of silver in the treatment of nervous diseases originated with the Arabs, probably from the influence of astrology in the medicine of that period. It was thought that nervous diseases were especially affected by the phases of the moon, which was associated with silver in their system, hence the names lunar caustic, lunacy, etc.

In the Indian indigenous system *Raupya Bhasma* is not only used against inflammation of the mucous membranes, but also in the treatment of neuritis and neuralgia. It is believed to have a soothing effect on the nerves including the peripheral nerve-endings. It is administered by the mouth in combination with different correctives which differ for different diseases. It is seldom used alone but is often used in combination with iron, tin, and gold. Modern research has shown that silver is not absorbed in sufficient quantity from the alimentary canal to produce any systemic effects. Its use in various diseases, therefore, has been entirely given up. Long continued use has been shown to produce blackish discoloration of skin called argyria due to the deposition of silver particle, probably in organic combination. Traces must therefore be absorbed, but these become fixed in the tissues in inert form.

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## SOME INORGANIC PREPARATIONS OF INDIAN INDIGENOUS MEDICINE.

### Part V.

#### SWARNA BHASMA (REDUCED GOLD) AND GOLD KUSTH.

BY

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GOLD as a metal has been known in India from time immemorial. Early works on old Hindu medicine show that a number of preparations have been used in therapeutics for many centuries. Pure gold leaf or gold free from admixture of dust, copper, silver, and other metals was usually employed, though in some preparations the metal was administered in the form of compounds. *Swarna Bhasma* is one of the commonest and a popular preparation. It is prepared in the following manner :—

The gold leaf is subjected to special treatment that goes by the name of 'purification'. The leaf is burnt in fire and the red hot metal is steeped seven times in each of the following : oil, whey, cow's urine, kanji, and extract of *Kulatha Kalai* (*Dolichos uniflorus*). There is more than one method of preparing the *bhasma* of which the following represent but a few : (1) In reducing gold one part of the purified metal and two parts of mercury are rubbed together with an acid and made

into a ball. Powdered sulphur equal in weight of the ball is taken ; half of the sulphur is placed in an earthen plate, the ball is placed over it and it is covered with the remaining half of the sulphur. The plate with its contents is covered with another earthen plate. A piece of rag is then smeared with clay and it is wrapped round the plate and dried in the sun. It is then placed on 30 pieces of dry cow-dung cakes and roasted. The process is repeated 14 times when the gold is converted into the *bhasma* form and is ready for use.

(2) Gold is reduced to a fine powder by rubbing with mercury and exposing it to heat in a covered crucible with the addition of sulphur. Two parts of mercury and one part of purified gold leaf are rubbed together into a mass with lemon juice and three parts of sulphur. The crucible is then covered and exposed to heat. The process of mixing gold with mercury and exposing the mixture, so formed, to heat is repeated 14 times when the gold completely loses its apparent metallic characters. Some are of opinion that gold should be rubbed with mercury on the first time of roasting and subsequent roasting should be done with sulphur alone.

(3) Another process of preparing reduced gold is that gold is melted and its own weight of ash of mercury is thrown into the molten metal. When cooled the mass is powdered and rubbed with lemon juice and cinnabar and again roasted in a covered crucible. The process is repeated 12 times.

By whichever process gold is reduced the principle underlying the preparation is that it should be intimately mixed with mercury, sulphur, and citric acid, and the mixture should be roasted several times. The reduced gold appears to undergo very little change from its metallic state, for on being rubbed on an agate mortar it produces a brilliant yellow stain like that of massive gold when the latter is rubbed on the touchstone for ascertaining its purity.

A sample of *Swarna Bhasma* obtained from the Kalpataru Ayurvedic Works, Calcutta, was analysed. The sample was a dull-brown amorphous powder with a metallic taste. On rubbing it over a hard surface it glistened and the physical character of gold was revealed. The chemical composition found as the result of our qualitative and quantitative analyses is as follows :—

					Per cent.
Gold, metallic	..	..	..	..	96·760
Silica, $\text{SiO}_2$	..	..	..	..	1·140
Iron, $\text{Fe}_2\text{O}_3$	..	..	..	..	0·140
Lime, $\text{CaO}$	..	..	..	..	0·546
Copper	..	..	..	..	traces.
Magnesia	..	..	..	..	traces.
Phosphates, $\text{P}_2\text{O}_5$	..	..	..	..	0·781
Potash, $\text{K}_2\text{O}$	..	..	..	..	0·161
$\text{NaCl}$	..	..	..	..	0·078
Sulphates, $\text{SO}_3$	..	..	..	..	0·150
Moisture	..	..	..	..	0·244
TOTAL					100·000

From this it appears that in the compound that we analysed gold was mostly present in the metallic state. Some oxide and sulphide of gold might be supposed to be present but owing to the instability of these compounds it is very difficult to accept such a conclusion, and even if they be present in it their quantities are obviously too small to be taken into consideration.

*Gold Kusth.*—This is a preparation of gold used in the Mohammedan medicine and the specimen examined was obtained from a well-known Hakim of Delhi and had the reputation of being a wonderful nerve tonic. It was a greyish amorphous



powder, insoluble in water. Qualitative tests showed that the whole of the gold contained was in a metallic state. On quantitative analysis it was found to contain 86.14 per cent of metallic gold. The other inorganic constituents, which amounted to 13.86 per cent, of the material could not be analysed in detail as the total amount sent for analysis was very small.

### *Pharmacology and therapeutics.*

Being an insoluble inorganic preparation, the pharmacological action of *Swarna Bhasma* and *Gold Kusth* could not be tested in the usual manner. These preparations consist mainly of metallic gold in a state of fine subdivision together with very small amounts of other compounds. The mode of administration of these preparations of gold, in the indigenous system, consists in rubbing this powder in a mortar along with correctives that differ in different diseases and then it is taken by the mouth. The paste so prepared is composed mainly of metallic gold in a fine state of division. Such a treatment probably partly converts the insoluble powder into the colloidal state and it is possible that this colloidal gold is taken up by the system in minute quantities and produces effects in neurasthenia and other nervous affections. It is also possible that metallic gold in this form is acted upon in the gastro-intestinal tract and may become converted into soluble compounds.

Soluble salts of gold, such as gold chloride, gold bromide, and potassium aurocyanide, have been used in modern medicine in a variety of diseases. Thus chloride of gold has been used both in the pure form and in combination with hydrochloric acid or sodium chloride, mainly in tubercular infections. The results are, however, still controversial, but it is believed in certain quarters that gold is effective in the early stages of tuberculosis when the infection is not intense. Gold bromide has been found to be very useful in epilepsy, while the potassium aurocyanide is believed to be useful in syphilitic infections. Besides these uses of the soluble salts of gold, colloidal gold has recently come into therapeutic use. Colloidal preparations have proved useful particularly in epilepsy, alcoholic neurasthenia, and in the morphine habit (Stanford, 1924). Gold if taken internally in the metallic state produces toxic symptoms resembling those of arsenic. But in the colloidal state it has been found to be beneficial to the system.

According to a well-known Ayurvedic physician of Calcutta *Swarna Bhasma* never produces any toxic symptoms. This is no doubt true as in the form in which it is present only very minute traces could be absorbed. It is believed to be a wonderful alterative and a tonic for the nervous system. It is an antidote to poisons, particularly those of bacterial origin. It is especially indicated in chronic fevers, tuberculosis, and neurasthenia. The dose varies from  $\frac{1}{4}$  grain to 1 grain but doses as large as 2 grains are administered. In the Hindu medicine gold in the form of *Bhasma* is believed to be a sovereign remedy in heart disease and as a general tonic in anæmia and debility. It has been recommended in various forms of dyspepsia with pain in epigastric region and looseness of the bowels. Gold is also considered to be a powerful sexual stimulant and to act beneficially in impotency. It has also been used in excessive nocturnal emissions in those who masturbate. In the Mohammedan medicine *Gold Kusth* or gold in form of leaf is used for similar purposes. Gold beaten down to the very thin consistency, less than that of thin

paper, is commonly used by people in such conditions. The effects produced may be partly psychical, but it is possible that minute quantities are absorbed and like some of the metals have a stimulating action on the metabolism as a whole.

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## GASTRIC ANALYSIS IN INDIANS.

### A STUDY OF 100 CASES.

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#### INTRODUCTION.

THE range of variation of gastric acidity, gastric motility, and gastric secretion in healthy individuals has been studied by many, and a 'standard' for the normal established. Rehfuess (1927) in America and Bennett and Ryle (1926) in England have established the standard in their countries. But we could not, from the literature available, get at any standard worked out in Indians. The tropical climate, the rich carbohydrate diet, and the different modes of living, may all influence the gastric function in more ways than one, so that it is possible that in people of a tropical country like India there may be a difference in the gastric secretion and motility when compared with those reported and established. Recently, Napier and Das Gupta (1935) have attempted to work out the gastric analysis in healthy Indians. That is, to our knowledge, the first one of its kind published in our country, though casual mention is made by some regarding the subject on the basis of a study of a few cases. Even in the paper above referred to, the authors mention that the investigations could not be done under ideal conditions on account of the following difficulties:—

1. 'It was impossible to persuade the coolies to desist from chewing betel-nut before the test.'
2. 'Few of them took the full quantity of the gruel.'
3. The Ryle's tube could not be retained till the stomach was emptied as 'samples of gastric juice were withdrawn as long as the subjects could be persuaded to retain the tube'.

Such errors were avoided in the present investigation which was carried out to determine 'the normal range' in a hundred healthy individuals.

## MATERIAL.

The cases have been selected mainly from the Ophthalmic Hospital of the Vizagapatam Medical College. The cases that were selected were all such that the complaints with which they came to the hospital did not necessitate their being kept in bed. They were not suffering from any symptoms referable to the digestive system and their complaints were such that a gross vitamin deficiency could be excluded. Such patients as had 'good appetite and digestion' only were selected for the purpose. Co-operation from the patients was obtained under the pretext that a fractional test-meal is an essential part of the treatment of the eye condition. The patients of all ages with the maximum possible variation from 12 to 75 years were taken, as it was shown by Alvarez *et al.* (1931) after an analysis of 3,746 records that free hydrochloric-acid values vary from childhood to youth and from youth to old age; of the 100 cases, 98 were males and 2 females. All those cases, who were suspected of having eaten, drunk, chewed, or smoked since 12 o'clock midnight, were excluded. Cases unable to retain the tube for  $2\frac{1}{2}$  hours or till the stomach was emptied were discarded.

## TECHNIQUE.

The patient was not permitted to take any solid or liquid food, nor allowed to chew or smoke after the previous night's dinner and the test-meal commenced between 9 a.m. and 10 a.m. the following morning.

The tube used for aspiration is a modification of Ryle's, having a rubber tubing of 4.5 mm. diameter with a hollow cylindrical gold tip, with its end rounded off and provided with four windows 2 mm.  $\times$  5 mm.

The gruel was made with one tablespoonful of barley powder in 600 c.c. of water boiled for about 15 minutes. After aspirating the resting juice from the stomach with an all-glass 10 c.c. syringe, the gruel was given by mouth. At intervals of every 15 minutes 20 c.c. of the gastric contents were aspirated till the stomach was emptied. During aspirations, it was attempted to keep a constant length of the tube in the stomach. The aspirated portions were filtered through a filter-paper. One c.c. of the filtrate was used for estimating the total chloride and 10 c.c. for estimating the free and total acids. The free and total acids were estimated by titration against  $N/10$  sodium hydroxide with Toepfer's reagent and phenolphthalein as indicators. The total chlorides were estimated by precipitating with excess of standard silver-nitrate solution in presence of strong nitric acid and back titration of the excess with standard potassium-thiocyanate solution.

In eleven cases the chlorides in blood were estimated immediately before and immediately after the test.

In six cases the pepsin content of the sample fractions was determined with a view to elucidate the relative relationship between the pepsin and other components of the gastric juice. The peptic activity was determined by Neirenstein and Schiff's modification of Mett's method. The Mett's tubes were prepared by a modification of Christiansen's method. Instead of sucking the albumin into the capillaries, which inevitably gave rise to minute air bubbles in the column of

albumin, the capillary tubes were slowly dipped into a narrow test-tube containing the albumin so that the capillary tube was filled with the albumin by capillary action. All the tubes were prepared at the same time and kept in stock for subsequent use. Briefly, the method consists in introducing the prepared capillary into the solution to be tested and kept for a definite length of time in the incubator. The protein column is digested at both ends of the tube to an extent depending on the amount of pepsin present. Neirenstein and Schiff showed that human gastric juice contained inhibitory substances, the effect of which is overcome by the dilution recommended. The peptic power is expressed as the square of the number of millimetres of albumin digested according to the Schutz Borrisow law.

For example, if the average of the four microscopic readings of the digested pepsin in the two capillary tubes digested by each c.c. of the gastric juice diluted sixteen times with 15 c.c. of N/20 HCl at 37°C. in 24 hours is 2.2 mm., the value for the diluted juice is  $2.2^2 = 4.84$  and for the pure undiluted juice  $4.84 \times 16 = 77.44$  units.

In each of the cases the history of the patient is noted as regards the age, occupation, nature of the diet, etc. (the diet and the habits of the people are discussed separately in another communication).

#### RESULTS.

*I. Resting juice.*—The amount of resting juice varied from a maximum of 95 c.c. to a minimum of 2 c.c.

Table I shows the variation of the amount of resting juice arranged in units of 15 c.c. :—

TABLE I.

Serial number.	c.c. resting juice.	Percentage of cases.
1	0-15	34
2	15-30	26
3	30-45	24
4	45-60	6
5	60-75	6
6	75 and more	4

The average amount of resting juice present was 30 c.c.

*II. The acidity.*—Table II shows the distribution of free HCl in the total number of cases. The maximum free acid recorded was 65 c.c. N/10 and the minimum zero :—

TABLE II.

c.c. N/10 acid (in units of 10 c.c.).	Total number of cases.
0-10 .. ..	28
10-20 .. ..	13
20-30 .. ..	12
30-40 .. ..	13
40-50 .. ..	7
50-60 .. ..	4
60-70 .. ..	3

*III. Other constituents.*—In no case was there found any residual starch in the resting juice. Bile was present in 22 cases. In some cases the resting juice was so viscid as not to permit more than 1 c.c. to filter through, which was utilized for estimating the total chlorides. The average amount of pepsin in the six cases was 224 units.

## SUBSEQUENT SPECIMENS.

*Acidity.*—The first specimen after the resting juice showed in nearly all the cases the lowest value for both the free and total acid: the free acid variations being from zero to 30 c.c. N/10. Then the acidity steadily increased in the large majority of cases and reached the maximum in  $\frac{1}{2}$  hour in 11 cases; in  $\frac{3}{4}$  hour in 22 cases; in 1 hour in 22 cases; in  $1\frac{1}{4}$  hours in 25 cases; in  $1\frac{1}{2}$  hours in nine cases; in  $1\frac{3}{4}$  hours in four cases; in 2 hours in three cases; in  $2\frac{1}{4}$  hours in two cases; and in  $2\frac{1}{2}$  hours in two cases. The maximum free acid reached was 86 (c.c. N/10). Table III shows the maximum free acid reached in various cases:—

TABLE III.

Maximum free acidity reached in terms of units of 10 c.c. N/10.	Number of cases.
0-10 .. ..	12
10-20 .. ..	18
20-30 .. ..	17
30-40 .. ..	17
40-50 .. ..	13
50-60 .. ..	8
60-70 .. ..	8
70-80 .. ..	5
80-90 .. ..	2

In five cases (Nos. 15, 17, 28, 65, and 71) the free hydrochloric acid was totally absent; of these five cases two showed normal chloride content (Nos. 95 and 115) and three showed subnormal chloride content of less than 70. In two other cases the free hydrochloric acid was practically nil, except for a slight rise of 1 to 2 c.c. N/10 in the later specimens. In these two cases (Nos. 87 and 94) the chloride content was 85 and 92.5 respectively. In all the seven cases the total acid curve was running close to the *x*-axis.

The difference between the total and free acids in all the cases was less than 10 c.c. N/10.

*Motility.*—The stomach was empty in 70 per cent of the cases between  $\frac{3}{4}$  and  $1\frac{1}{4}$  hours. Table IV shows the emptying time in the various cases:—

TABLE IV.

Emptying time in hours.	Percentage of cases.
$\frac{1}{2}$ ..	9
$\frac{3}{4}$ ..	21
1 ..	20
$1\frac{1}{4}$ ..	29
$1\frac{1}{2}$ ..	11
$1\frac{3}{4}$ ..	3
2 ..	3
$2\frac{1}{4}$ ..	2
$2\frac{1}{2}$ ..	2

The average emptying time was  
1.14 hours or 1 hour 8 minutes.

*Total chlorides.*—The total chlorides of the gastric contents were estimated in nearly all the cases. The initial resting juice showed the chloride content to be from 55 c.c. to 140 c.c. N/10 per 100 c.c., the average being 96.62 c.c.; the majority of cases were found to have a chloride value between 90 and 100.

*Pepsin* by Mett's method (as described before) was estimated in six cases. Table V represents the pepsin value in the six cases along with the total chloride and free hydrochloric-acid content. It is clearly seen that the changes in these constituents parallel one another :—

TABLE V.

Serial number.	Case number.	Aspirated specimen number*.	Pepsin (units).	Chloride (c.c. N/10).	Free acid (c.c. N/10).
1	72	R	231	110	17·5
		1	20	20	5
		2	36	25	11·5
		3	64	37·5	18·5
		4	92	47·5	26
		5	150	77·5	32·5
2	73	R	442	105	56·5
		1	125	27·5	14
		2	339	45	30
		3	288	60	45
		4	384	75	..
3	77	R	243	115	..
		1	52	20	10
		2	58	42·5	20
		3	168	97·5	..
4	78	R	81·6	105	46
		1	36	37·5	16
		2	64	50	22·5
		3	72	67·5	40
		4	144	87·5	55
5	79	R	121	95	25
		1	16	15	1·5
		2	41	30	14
		3	109	55	35·5
6	80	R	..	72·5	..
		1	..	17·5	..
		2	48	22·5	..
		3	144	52·5	18
		4	72	50	14·5
		5	163	90	42
		6	134	70	25

\* R represents the resting juice and the subsequent figures the subsequent specimens, aspirated at intervals of 15 minutes.



*Blood chlorides.*—The chloride content of the blood was estimated in 11 cases immediately before and immediately after the gastric analysis. Table VI represents the results along with the corresponding gastric chloride values.

The chloride values are represented in terms of  $N/10$  chloride-ion.

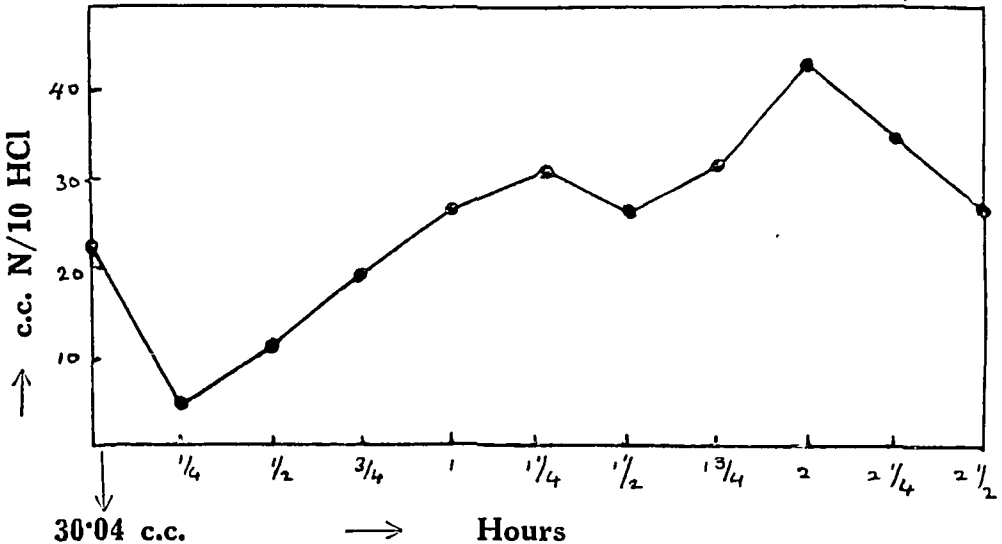
TABLE VI.

Serial number.	Case number.	CHLORIDES BEFORE GASTRIC ANALYSIS.		CHLORIDES AFTER GASTRIC ANALYSIS.	
		Blood.	Gastric.	Blood.	Gastric.
1	68	103	102.5	89.6	82.5
2	69	85.8	110	78.9	47.5
3	70	90.3	105	93.7	45
4	72	89.6	110	95.5	77.5
5	73	83.7	105	84.8	75
6	74	80.6	120	54.1	97.5
7	75	97.6	127.5	97.9	92.5
8	77	88.9	115	..	97.5
9	78	85.8	105	89.6	87.5
10	79	89.2	95	91.3	55
11	80	86.2	72.5	92.4	70
Average ..	..	89.10	106.1	86.8	75.2

## REVIEW.

The composite curve of the free acid values is given in Graph 1 :—

GRAPH 1.



The variation in the free acid content of the gastric juice at the various stages of the fractional test-meal is recorded in Graph 2. Graph 3 represents the range of variation in 80 per cent of these cases. Graphs 4 and 5 represent the corresponding ones given by Bennett and Ryle (*loc. cit.*). While comparing the two it may be pointed out that while the European and American authors worked on students, our cases were picked from the labouring classes.

A comparison of Graphs 2 and 3 with Graphs 4 and 5 brings to light the following facts :—

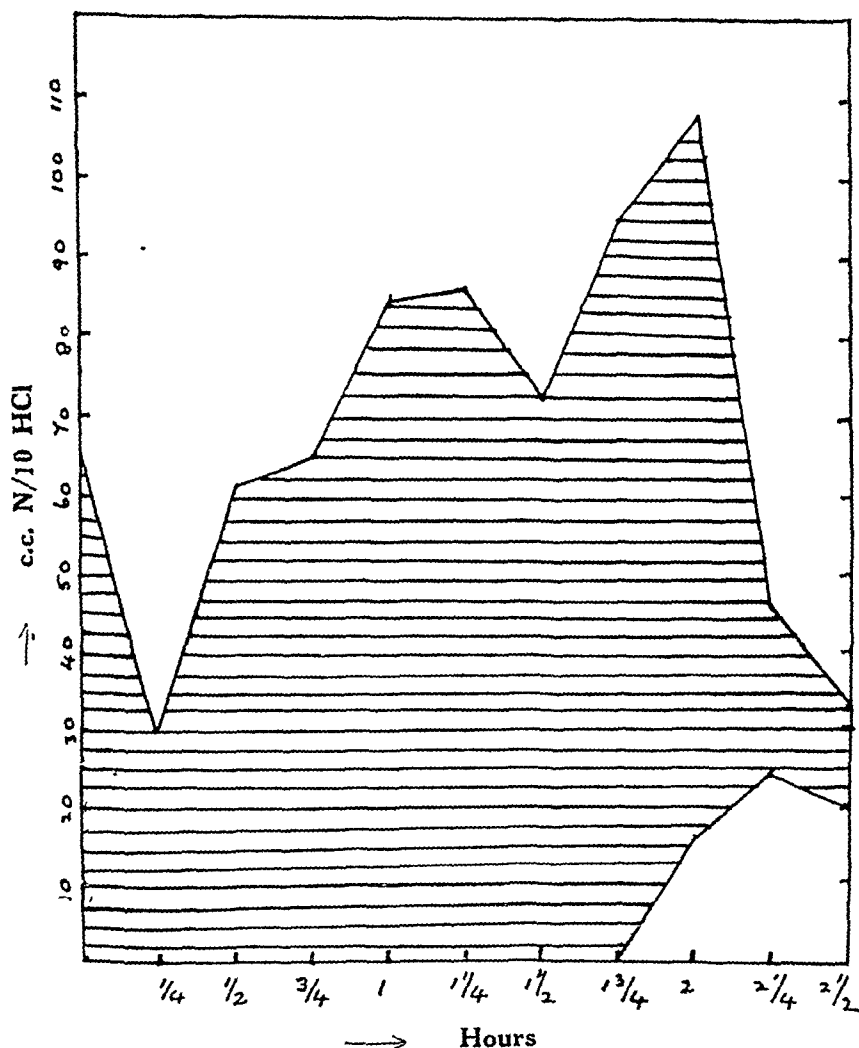
**Acidity.**—The initial acidity ranges from 0 to 23 in 80 per cent of Ryle's series, whereas in the present series in 80 per cent of the cases the range is between 0 and 50 c.c. N/10. The maximum acidity is reached in  $1\frac{1}{2}$  hours in Ryle's series, the maximum being only 47 c.c. N/10. The corresponding figures in the present series are 61 c.c. N/10 in  $1\frac{1}{4}$  hours.

Some of the individual cases deserve mention. In two cases (Nos. 91 and 61) the free acidity and the motility were such as to resemble the curves usually found in cases of chronic duodenal ulcer with pyloric obstruction, whereas in some others the initial high acidity was so high and the emptying so rapid that they looked pathological. These findings only bring home the facts that hyperchlorhydria in a definite though small number of cases is normal and that test-meal analysis forms only an auxiliary factor in diagnosis.

On the other extreme of the results seven cases may be said to be achlorhydric but (I) taking into consideration that true achlorhydries should show low total

chloride values also and (11) discarding the last two cases which showed complete absence of free hydrochloric acid till the fifth specimen only, but showed a slight rise of 2 c.c.  $N/10$  in the last two specimens, the percentage figure of pure achylia may be taken only as 3. Probably in the discarded four cases the achlorhydria is

GRAPH 2.

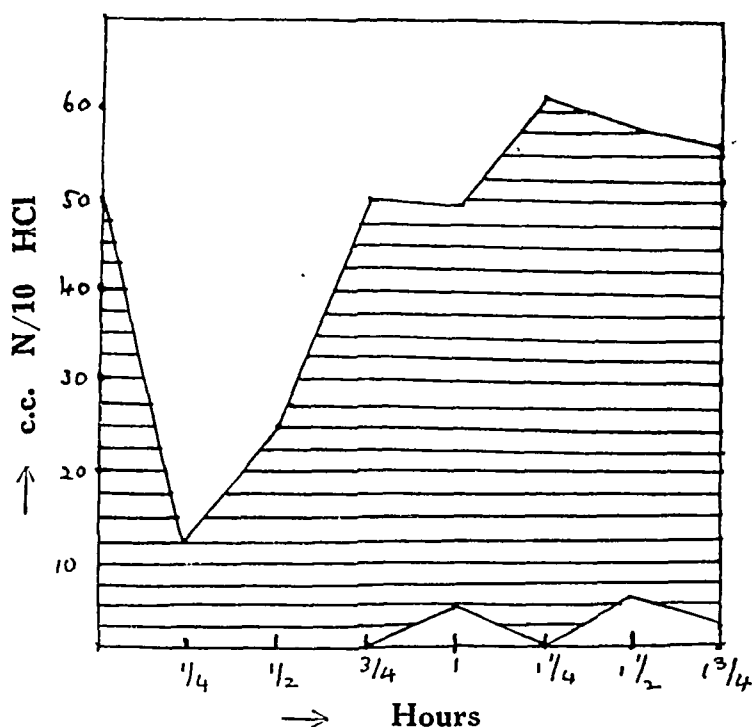


brought about by excessive neutralization as pointed out by Campbell (quoted by Bennett and Ryle, *loc. cit.*). The 3 per cent of the achylia cannot be contrasted with the 4 per cent of Ryle's series, as in those four cases total chlorides have

not been calculated. In this connection it may also be recalled that our finding is not in accordance to that of Napier and Das Gupta, who claim, in their series of forty-three, only one case of achlorhydria (true or pseudo, not mentioned).

*Motility.*—The majority of cases was empty within  $\frac{3}{4}$  hour whereas in Ryle's series they were within  $1\frac{3}{4}$  hours. Moreover, in 80 per cent of the cases the stomach was not empty till  $2\frac{1}{2}$  hours, whereas in the present series more than 80 per cent of the cases were empty within  $1\frac{3}{4}$  hours.

GRAPH 3.

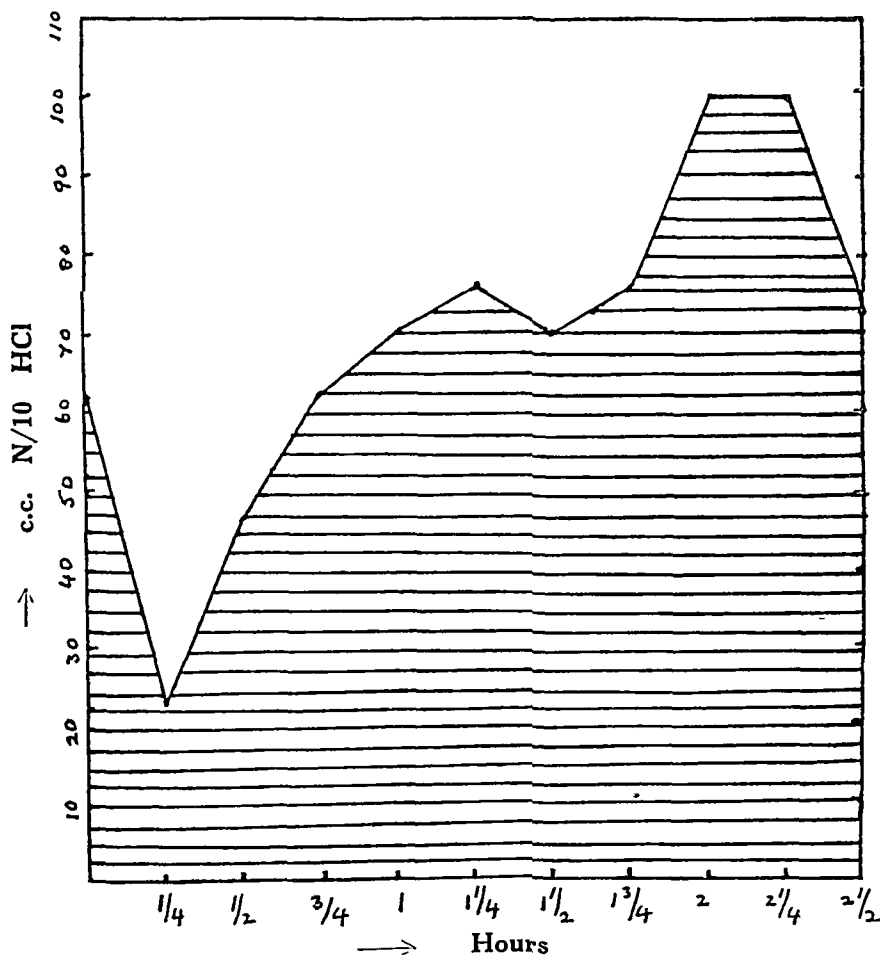


*Pepsin.*—The pepsin content in the small number of cases (Table I) is proportionate directly to the free acid and total chlorides. The average pepsin content of the gastric juice is said to be about 100 units (Rehffuss, *loc. cit.*). But the results in the six cases show that in the residual juice the pepsin content of 224 units is far higher.

*Comment.*—McLean's (1928) view of gastric secretion, as the total gastric acidity being directly dependent on the blood chlorides, has been contradicted by some. Judging from the present results (Table VI) one can only conclude that gastric chlorides and blood chlorides approximate each other only to a certain extent as digestion proceeds. This suggests that, in addition to the fact that concentration of the chloride-ion being constant in the blood and gastric juice at the same time in an isolated pouch, there are some other factors responsible in controlling the acidity in an intact stomach during actual digestion.

The maximum and minimum limits in acidity in the present 100 cases are found to be more or less identical with that of Ryle, but the range of dispersion seems to vary in the two series. The normal range of variation in acidity in 80 per cent of the cases is found to be different in many ways from that reported by Ryle. The range of initial acidity is more, the maximum acidity is higher, and

GRAPH 4.



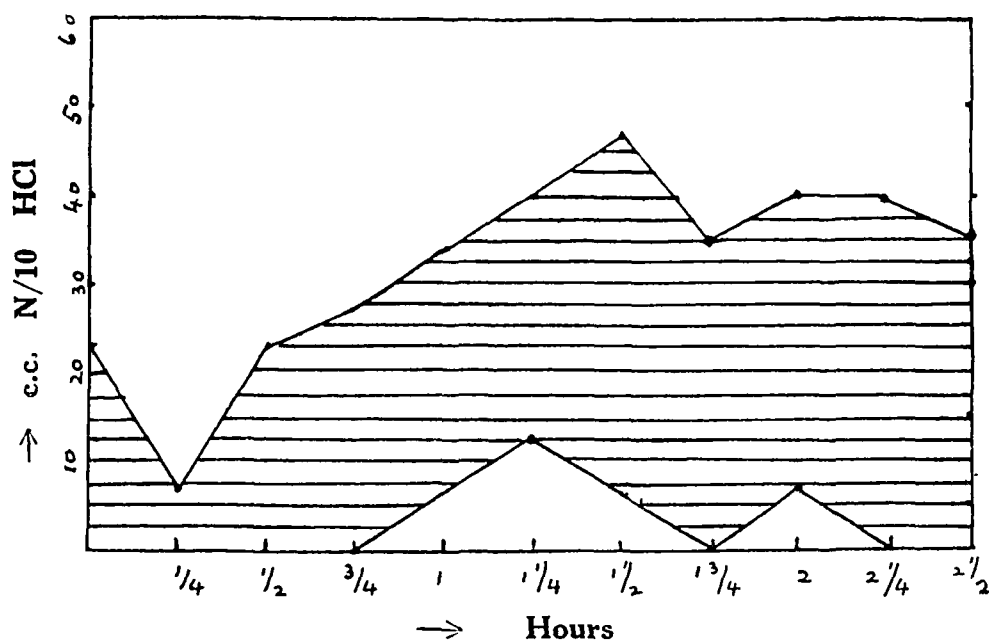
reached earlier in the course of the analysis. The motility also is greater and the emptying time less. The pepsin content is also higher than the supposed average.

It is difficult to detail the factors responsible for the higher acidity and hypermotility in the present series. Probably the diet, rich in carbohydrate, condiments, and spices, and poor in vitamins A and B, which are the salient features in the diets

of these people (as will be pointed out in a separate communication), is to a large extent responsible. The normal stomach which secretes a hyperacid juice may incidentally be prone to diseases predisposed by hyperacidity. The chemical factor in the ætiology of peptic ulcer cannot be gainsaid. Given a susceptible tissue in a person with gastric ulcer diathesis, a hyperacid gastric juice does precipitate the formation of an ulcer. Many an example may be cited to illustrate the same, as it is not uncommon to find a jejunal ulcer forming when an anastomosis is done with a hyperacid stomach.

The relative greater incidence of duodenal as compared to gastric ulcers in these areas (13 to 26 : 1) when compared with figures from foreign clinics (1 to 8 : 1) may also be accounted for by this fact. Peptic ulcers in certain instances form at the junction of the tissues bathed in acid and alkaline media respectively. It is

GRAPH 5.



not uncommon to find a peptic ulcer at the verge of an aberrant gastric mucosa in a Mackel's diverticulum. It is also not uncommon to find an ulcer developing at the suture line when the acid gastric juice is diverted to the alkaline lower ileum in operations like 'the duodenal drainage', i.e., the ulcers have a tendency in some instances to form at the junction where the acid juice is neutralized by the alkaline juice and the greater acidity in the stomach would deviate this 'point' of neutralization farther and farther distally, so that there is a greater likelihood of the ulcer forming in the duodenum.

#### SUMMARY.

1. Gastric analysis by the method of fractional test-meal has been done in a hundred healthy South Indians, the various factors analysed being the free

hydrochloric acid, the total acid, and the total chlorides. The pepsin content and the blood chlorides also were estimated in some cases.

2. A chart showing the variation of free hydrochloric acid of the gastric contents in 80 per cent of healthy South Indians is given,

3. The probable relation between the higher normal acidity and the greater incidence of duodenal ulcer in the Circars is discussed.

#### ACKNOWLEDGMENTS.

I take this opportunity to thank the Principal, Medical College, Vizagapatam, and the Professor of Biochemistry for permission to work in the college laboratories ; Dr. V. K. Narayana Menon, under whose direction the work was undertaken, for the valuable criticism in preparing the paper ; Dr. M. G. Kini for the helpful guidance ; Dr. C. Abbu, Dr. N. G. Gantayet and other medical officers, who kindly supplied the clinical material for the work.

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## HÆMATOLOGICAL STUDIES IN INDIANS.

### Part VII.

#### THE INCIDENCE AND DEGREE OF ANÆMIA AMONGST PREGNANT FEMALES OF THE COOLIE POPULATION.

BY

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AND

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*(An Inquiry under the Endowment Fund of the School.)*

*(From the School of Tropical Medicine, Calcutta.)*

[Received for publication, December 31, 1936.]

As a preliminary measure to a more detailed hæmatological investigation of the anæmia occurring in pregnant women, we estimated the hæmoglobin of a sample of the pregnant women in the tea-garden coolie population.

It is a practice on many tea gardens in Assam to pay the women who are pregnant, during the period of their pregnancy, when they are incapacitated from performing their normal work; in order to get this pay they have to report at the hospital where they are sometimes given light duties suitable to their condition. This practice has the very great advantage of bringing the women under medical attention, and it provided us with an opportunity of estimating the hæmoglobin in an unselected group of pregnant women.

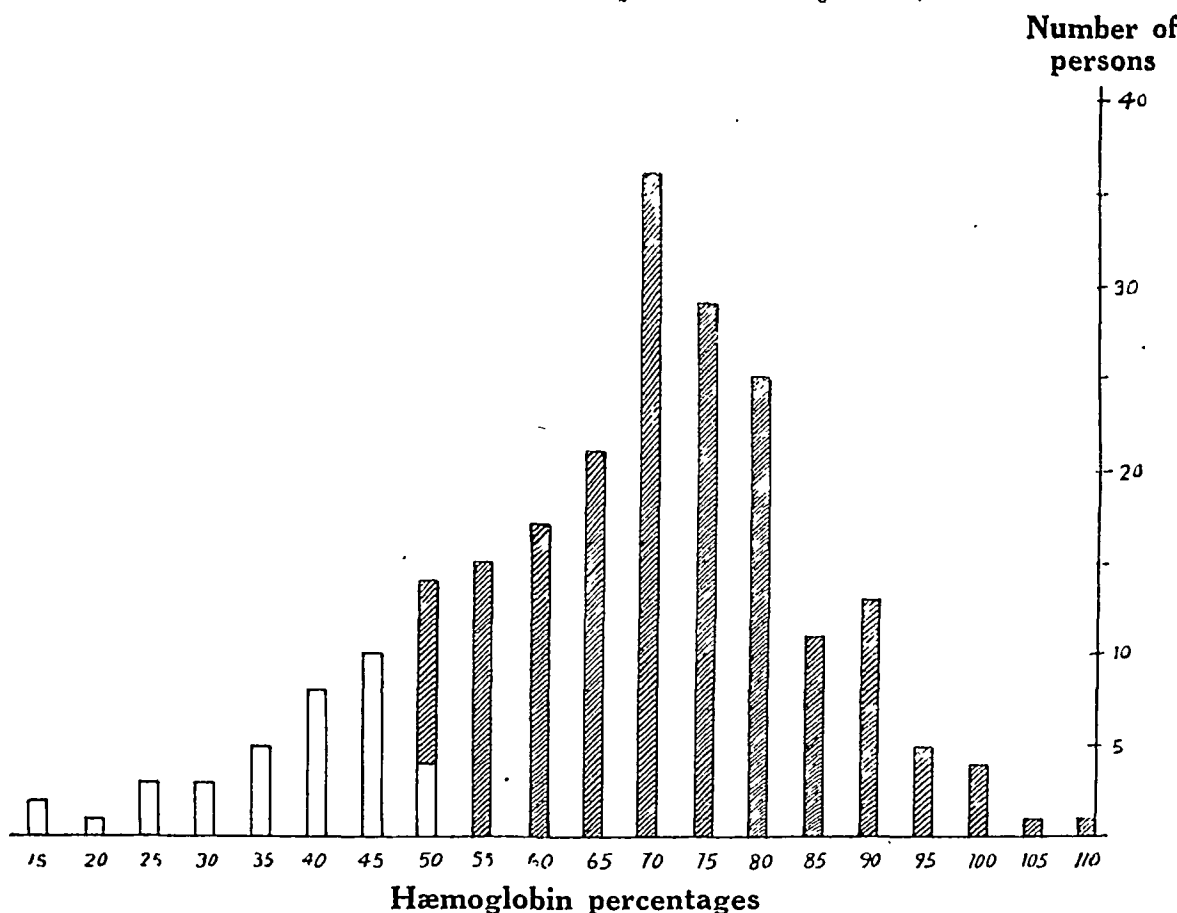
Samples were taken from 228 pregnant women on 19 different gardens; the estimations were made with the Hellige apparatus. The findings are summarized below.

The Chart shows the distribution of the women according to their hæmoglobin percentage (100 per cent = 13·75 grammes per 100 c.c.) in 5 per cent groups. There is no sharp line of distinction between the anæmic and the 'normal' women. The mean hæmoglobin of the whole series is 9·22 grammes; the figure is but distinctly lower than 10·03 grammes, the figure we call for coolie women (*vide* Part III), but to obtain this latter

obviously anæmic patients and arbitrarily took 50 per cent (6·875 grammes) as our lowest level for 'normality'; we have therefore done the same with the figures reported here, and have divided the women into two groups, 36 anæmic and 192 'normal' women. The mean and standard deviation of the 'normal' group is  $9\cdot99 \pm 1\cdot72$  grammes; this is remarkably near our figures for non-pregnant coolie women (i.e.,  $10\cdot03 \pm 1\cdot70$ ).

# CHART.

*Showing the distribution according to hæmoglobin percentages, in 5 per cent groups, of 228 pregnant women (100 per cent hæmoglobin=13·75 grammes per 100 c.c. of blood).*



We have no large series of figures of non-pregnant women that is strictly comparable with these\* as the incidence of anæmia of this order varies on different

\* The hæmoglobin estimations on tea-garden coolies reported in Part I of this study were designed originally to supply these figures, but through a misunderstanding this series was unfortunately vitiated, for this purpose, by the inclusion of selected anæmic cases from the rest of the population, on most of the gardens.

gardens, but it is not usually more than 10 per cent, whereas in this series it is 15·8 per cent. The evidence is therefore that whilst pregnancy tends to increase very slightly the incidence of anæmia it does not lower the hæmoglobin level of the female population to any appreciable extent.

Arranged according to the number of their pregnancies these women show percentage incidences of anæmia as follows :—

Pregnancy.	Number with anæmia.	Number 'normal'.	Percentage with anæmia.
First ..	8	20	28·5
Second ..	6	33	15·4
Third ..	8	52	13·3
Fourth ..	5	36	12·2
Fifth ..	3	17	9·6
Sixth ..	1	10	
Seventh ..	..	9	
Eighth ..	1	8	
Ninth ..	..	3	
Not noted ..	4	4	

Arranged according to age they show percentage incidences of anæmia as follows :—

Age group.	Number with anæmia.	Number 'normal'.	Percentage with anæmia.
15 to 19 ..	7	14	33·3
20 to 24 ..	8	40	16·7
25 to 29 ..	8	43	15·7
30 to 34 ..	8	59	11·9
35 to 39 ..	4	29	13·2
40 and over ..	1	4	
Not noted ..	..	3	

Arranged according to the month of pregnancy they show percentage incidences of anæmia as follows :—

Month.	Number with anæmia.	Number 'normal'.	Percentage with anæmia.
Ninth ..	11	46	19.3
Eighth ..	7	72	8.9
Seventh ..	10	33	23.3
Sixth ..	4	26	13.3
Fifth ..	2	9	21.1
Fourth ..	2	4	
Third ..	..	2	

It would appear from these tables that anæmia was far more frequent in the first pregnancy and that the incidence decreases with each succeeding pregnancy up to the fourth after which the data are too small for separate consideration.

Similarly, it would appear that anæmia is most common in the youngest age group.

The possibility of these two factors being connected is obvious, but it is not possible to say which is the more important one.

Statistically, the percentage incidence of anæmia is 'significantly' greater in the first pregnancy than in the pregnancies subsequent to the fourth, but the difference between that of the first pregnancy and of the second, third, or fourth, respectively, although considerable, is not 'significant'.

The percentage incidence of anæmia in the fourth quinquennial period (i.e., under twenty years) is 'significantly' greater than that in any of the subsequent periods.

On the other hand there seems to be no correlation between the incidence of anæmia and the duration of the pregnancy.

As this finding was a little surprising we decided to examine the figures in another way and to calculate the mean of the hæmoglobin values for different months of pregnancy, separately for the anæmics and non-anæmics.

Month of pregnancy.	MEAN HÆMOGLOBIN PERCENTAGE 100 PER CENT = 13.75 GRAMMES.	
	Anæmics.	Non-anæmics.
9 ..	41.2	78.21
8 ..	34.3	73.6
7 ..	32.9	72.9
6 ..	40.2	73.0
Less than 6 ..	38.5	74.0

These figures again seem to indicate quite clearly that there is no positive correlation between the length of duration of pregnancy and the hæmoglobin deficiency in these women. In fact, the figures suggest that such correlation as there is may be a negative one.

There is, however, a possible fallacy. The women usually first report themselves as pregnant during the 7th or 8th month; they are thenceforth under some medical supervision and the anæmic ones are usually placed under treatment.

To summarize, the hæmoglobin state of the general female population is far below that of other female populations, but there is little evidence of any general 'physiological' lowering of the hæmoglobin level in pregnancy that has been noted in other countries (Bethell, 1936), nor is there evidence of any progressive hæmoglobin deterioration throughout the course of pregnancy.

There is some evidence of a slightly higher incidence of definite anæmia amongst pregnant women than amongst the general female coolie population; the incidence of this anæmia is higher amongst the younger women and during the first pregnancy.

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## HÆMOLYSIS BY THE VENOM OF THE INDIAN COBRA (*NAJA TRIPUDIANS*).

BY

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THE hæmolytic action of snake venoms has been the subject of a considerable amount of study, notably by Kyes (1902), Kyes and Sachs (1903), and more recently by Kellaway and Williams (1933), and other workers. The general nature of this action in case of many venoms is recognized to be a lecithinase one, the lecithinase acting on the lecithin of the blood and more particularly at the limiting surface of the red blood cells producing a lyso-lecithin, by the separation of an unsaturated fatty acid from the lecithin molecule, to which the hæmolysis is due. A number of different factors appears to enter into hæmolysis and attention was drawn to certain of these in connection with work carried out originally for the purpose of determining in the case of cobra venom whether the neutralization of its hæmolytic action could be used as a method for titration of titre of cobra antivenene.

In a previous communication (Ganguly and Malkana, 1936) it has been shown that when the proteins of cobra venom were fractionated the neurotoxic action of the venom was found to be associated with the secondary proteoses. A further study of the protein fractions now carried out shows that the hæmolytic action of the venom is associated with the primary proteose and globulin fraction. These fractions are non-toxic but show the whole hæmolytic action of the venom. The results of tests of these fractions are given in Table I :—

TABLE I.

*Showing the hæmolytic action of cobra venom fractions on the red blood cells of different animals.*

Venom fractions used.	RESULTS OF HÆMOLYSIS ON ANIMALS USED (WITHOUT COMPLEMENT).			
	Horse.	Human.	Monkey.	Guinea-pig.
1. Primary proteose .. ..	++	++	++	++
2. Globulin and primary proteose ..	++++	++++	++++	++++
3. Secondary proteose .. ..	--	--	--	--
4. Albumin and secondary proteose ..	--	--	--	--
5. Saline control .. ..	--	--	--	--

As certain workers have attributed the hæmolytic action to the phospho-lipoids the tests were repeated with the same fractions after extraction of the phospho-lipoids with the results shown in Table II:—

TABLE II.

*Showing the hæmolytic action of the venom fractions after the extraction of the phospho-lipoids.*

Venom fractions used.	RESULTS OF HÆMOLYSIS ON ANIMALS USED (WITHOUT COMPLEMENT).			
	Horse.	Human.	Monkey.	Guinea-pig.
1. Primary proteose .. ..	++	++	++	++
2. Globulin and primary proteose ..	++++	++++	++++	++++
3. Albumin and secondary proteose ..	--	--	--	--
4. Phospho-lipoid .. ..	--	--	--	--
5. Saline control .. ..	--	--	--	--

*Note.*—The fraction 2 (globulin and primary proteose) represents 31·62 per cent of the total dried venom and the hæmolytic action of this fraction has been found to be relatively stable.



The presence of lecithinase in the globulin and the primary proteose fraction were first determined by testing hydrolysis of lecithin followed by titration of the unsaturated fatty acids produced, using the technique previously described (Ganguly, 1936). The following results were obtained :—

Venom fraction used.	The amount of 0.0188 N alkali required (in c.c.) after a reaction of 48 hours, for 5 c.c. of the reaction mixture.
1. Globulin and primary proteose ..	1.65
2. Cobra venom (untreated) ..	1.70
3. Secondary proteose and albumin ..	0.85
4. Saline control .. ..	0.80

The results show that lecithinase is associated with this fraction in some loose chemical combination or in an adsorbed state. It was found that within a range of pH 6.0 to pH 8.0 of the substrate no appreciable difference in hydrolysis occurred.

This hæmolytic fraction was employed for certain quantitative studies of factors concerned in cobra venom hæmolysis.

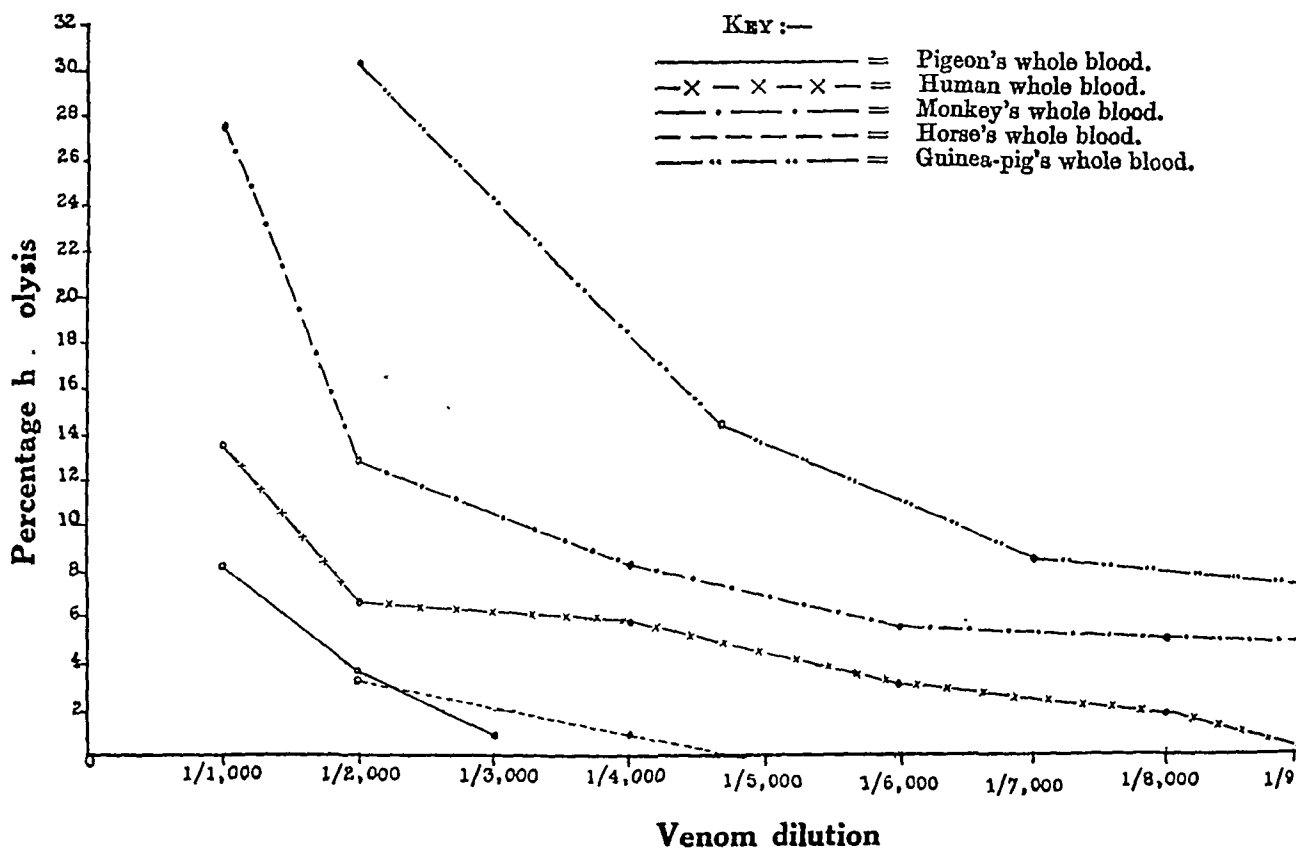
#### *Hæmolysis of blood of different species of animals.*

The following technique was employed :—

To whole citrated blood or to suspensions of washed cells of different species various dilutions of a solution of the hæmolytic fraction were added. Nine parts of blood or of cell suspensions were placed in small test-tubes and one part of the known dilution was added. A saline control in place of the hæmolytic fraction was also used for each series. The tubes were incubated at 37°C. for two hours. Varying degrees of hæmolysis were found to occur in this period according to the venom fraction dilution and the species of animal. The tubes were centrifuged and the supernatant fluid from those containing the hæmoglobin released from the cells was collected into separate flasks. A drop of pure hydrochloric acid was added and each was made up to a constant volume. The percentage hæmolysis was

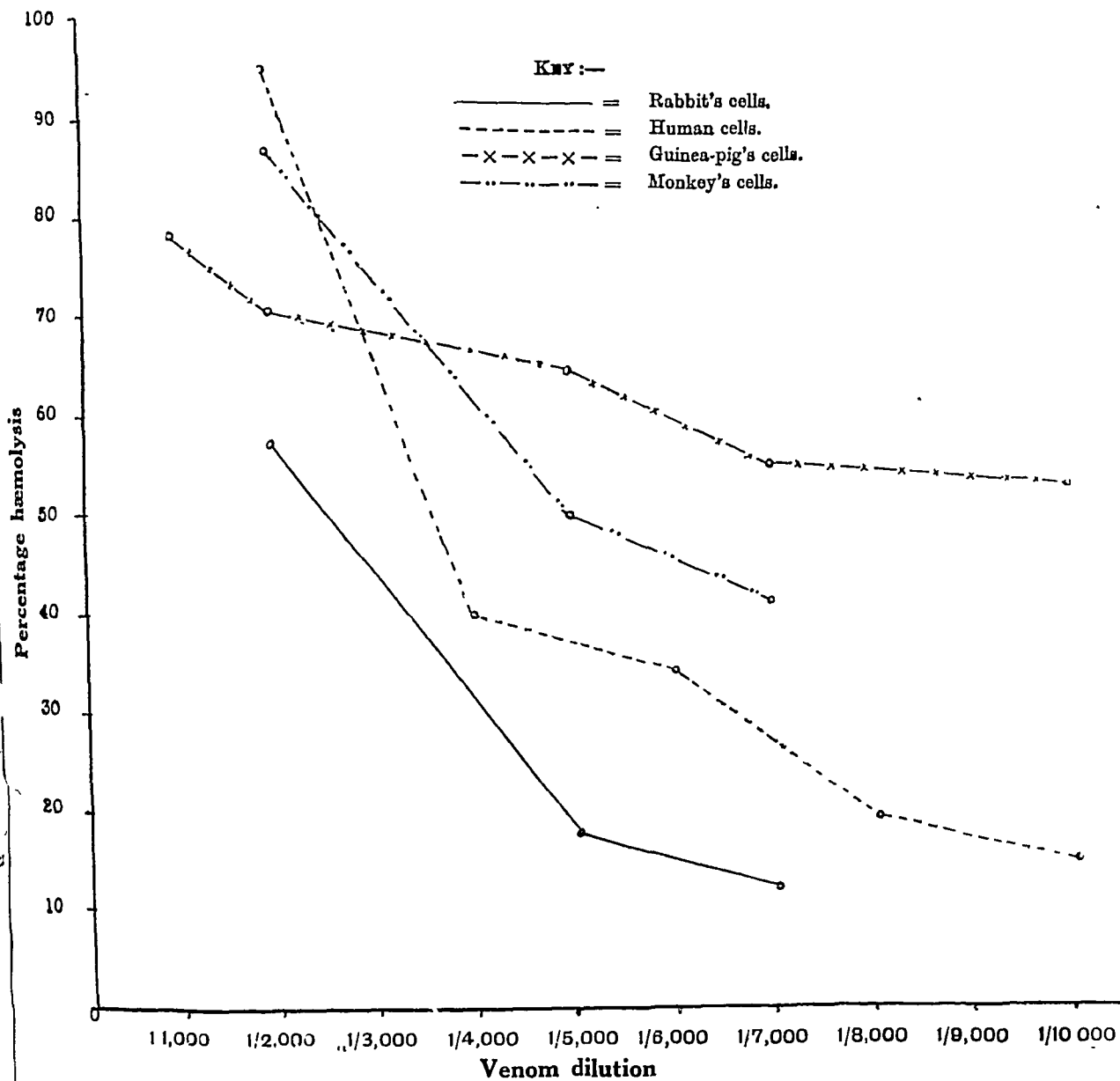
estimated in the colorimeter by comparison with a standard prepared from the saline control by hæmolysing it with HCl, this representing complete hæmolysis of the quantity of cells used in the test.

GRAPH 1.



The comparative hæmolytic action of the venom hæmolysin in different dilutions (given in relation to whole dried venom which it represents) on whole blood and also on 5 per cent cell suspensions of different species of animals is shown in Graphs 1 and 2.

GRAPH 2.



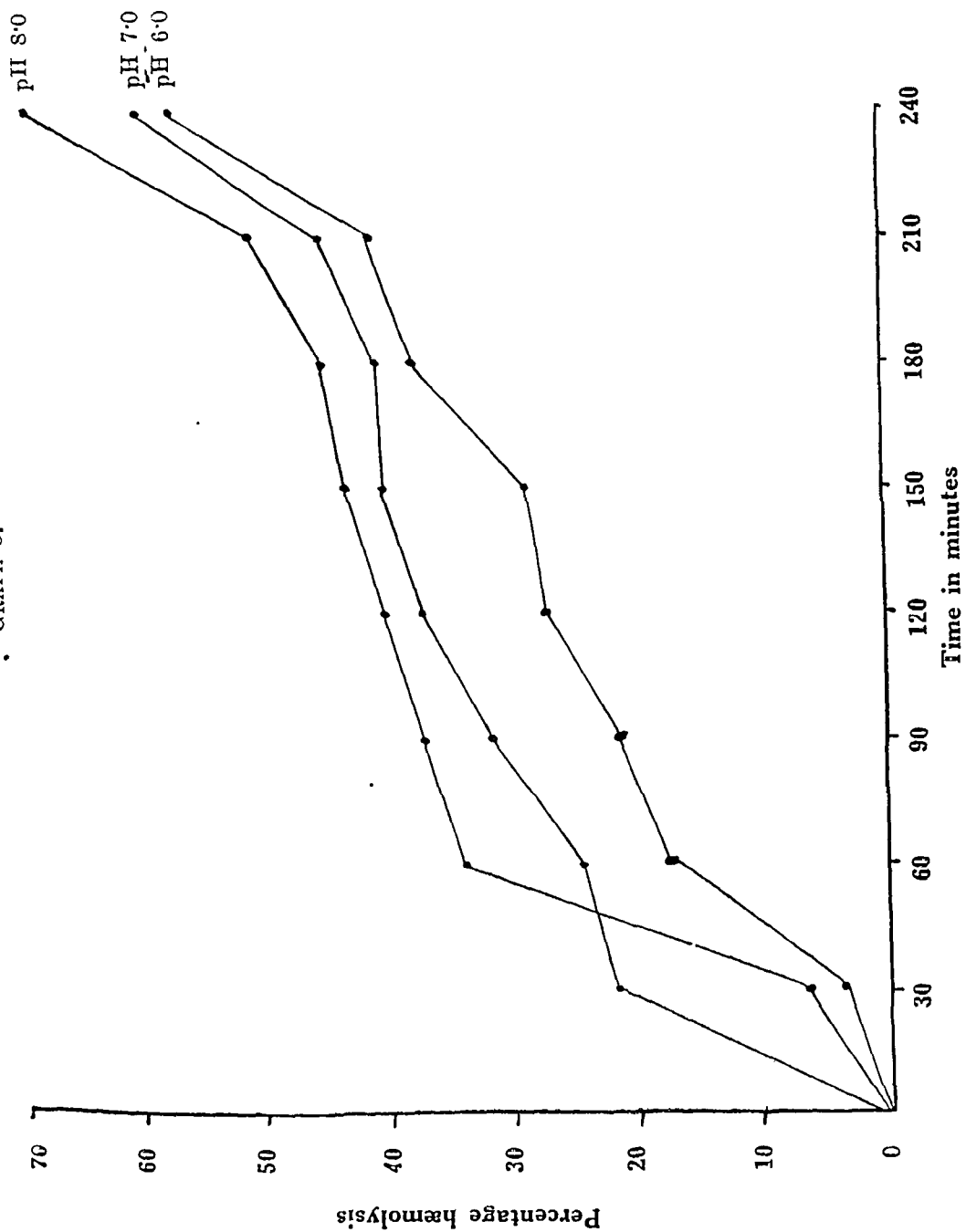
A dilution of 1 : 2,000 of the venom hæmolysin was found to show a marked differential action on 5 per cent suspension of cells and the whole blood of different species, and in further work this dilution only was used in most cases. The following figures will indicate the relative hæmolysis that occurred with this dilution:—

Species.	Percentage hæmolysis.
1. <i>Guinea-pig.</i>	
Whole blood ..	30·6
Cell suspensions ..	78·5
2. <i>Rabbit.</i>	
Whole blood ..	28·5
Cell suspensions ..	62·0
3. <i>Monkey.</i>	
Whole blood ..	13·2
Cell suspensions ..	86·9
4. <i>Human.</i>	
Whole blood ..	6·9
Cell suspensions ..	96·0
5. <i>Horse.</i>	
Whole blood ..	3·4
Cell suspensions ..	79·3
6. <i>Sheep.</i>	
Whole blood ..	<i>Nil</i>
Cell suspensions ..	<i>Nil</i>

Guinea-pig cells are the most sensitive to the hæmolytic action, while sheep cells are not hæmolysed under the conditions of the experiment. This resistance of the sheep cells has been noticed by many workers using other venoms.

Little difference was shown in the degree of hæmolysis in certain ranges of hydrogen-ion concentration used. Although the velocity of hæmolysis was somewhat different, the end figure was not much affected. The findings in this respect are very similar to those on lecithin hydrolysis by the cobra venom hæmolysin (Graph 3).

, GRAPH 3.



The lyso-lecithin produced by the action of cobra venom on lecithin can combine in equimolecular proportions with cholesterol (Cameron, 1933) and observations made during the course of the work on the neutralization of hæmolytic action with antivenene suggested that cholesterol played a part in preventing hæmolysis of greater importance than has been assigned to it by some workers.

*The neutralization of cobra venom hæmolysis by cholesterol.*

The neutralizing action of cholesterol on hæmolysis by cobra venom or its hæmolytic fraction acting on a fixed quantity of a lecithin substrate was tested on washed rabbit cells. The percentage hæmolysis with various mixtures was determined after two hours at 37°C. The results are shown in Table III. The addition of a quantity of cholesterol equal to that of the lecithin caused complete inhibition of hæmolysis by a dilution of cobra venom or the hæmolytic fraction which would without cholesterol produce about 60 per cent hæmolysis.

TABLE III.

*Showing the effect of variation of the lecithin and cholesterol content of the hæmolytic system on venom hæmolysis.*

Lecithin (0·5 per cent suspension) in c.c.		Cholesterol (0·5 per cent suspension) in c.c.	Cobra venom (1 per cent) in c.c.	Cobra venom hæmolysin (1·0 per cent) in c.c.	Percentage hæmolysis.
1.	0·9 ..	<i>Nil</i>	0·1	<i>Nil</i>	62·0
2.	0·9 ..	„	<i>Nil</i>	0·1	57·0
3.	0·9 ..	„	„	<i>Nil</i>	9·0
4.	0·9 ..	0·9	0·2	„	6·0
5.	0·9 ..	0·9	<i>Nil</i>	0·2	6·0
6.	0·9 ..	0·9	„	<i>Nil</i>	No hæmolysis
7.	<i>Nil</i> ..	0·9	0·1	„	„ „
8.	„ ..	0·9	<i>Nil</i>	0·1	„ „

*Note.*—0·5 c.c. of each of the mixtures shown in the table was separately mixed with 0·5 c.c. of 20 per cent cell suspensions of rabbit's cells and incubated at 37°C. for two hours before taking the readings.

Difficulty was experienced in carrying out corresponding tests by the addition of cholesterol to blood but the proportionate hæmolysis produced by the hæmolytic fraction was determined for different species and correlated with their normal content of both lecithin and cholesterol. Lecithin and cholesterol estimations

were carried out on whole blood and corpuscles of guinea-pig, rabbit, monkey, horse, sheep, and human beings, on the same specimens as were used for hæmolytic tests. Sackett's (1925) method was used for the estimation of cholesterol and Youngburg and Youngburg's (1930) method for lecithin. The results are shown in Table IV, which also shows the percentage hæmolysis obtained with a 1 : 2,000 dilution of the cobra venom hæmolysin after two hours' incubation at 37°C.

TABLE IV.

*Showing the relationship of percentage hæmolysis with lecithin and cholesterol content of whole blood and red blood corpuscles of different species of animals.*

Species.	Cholesterol in mg. per 100 c.c.	Lecithin in mg. per 100 c.c.	Percentage hæmolysis.
1. <i>Guinea-pig.</i>			
Whole blood ..	97.5	240.0	50.5
Cells ..	120.3	207.0	70.6
2. <i>Rabbit.</i>			
Whole blood ..	110.0	190.0	28.6
Cells ..	180.0	420.0	62.0
3. <i>Monkey.</i>			
Whole blood ..	200.8	166.3	13.2
Cells ..	151.6	190.9	86.9
4. <i>Human.</i>			
Whole blood ..	220.0	300.0	6.9
Cells ..	200.0	420.0	95.0
5. <i>Horse.</i>			
Whole blood ..	248.0	153.4	3.4
Cells ..	230.0	160.9	79.8
6. <i>Sheep.</i>			
Whole blood ..	333.5	202.4	<i>Nil</i>
Cells ..	518.0	153.4	<i>Nil</i>

It is seen that the degree of hæmolysis produced in whole blood is in inverse proportion to cholesterol content, although proportionate relationship between cholesterol and lecithin in this regard cannot be demonstrated.

The very high cholesterol content of sheep's blood and of sheep's corpuscles is striking and this factor is a possible explanation of the complete failure to produce hæmolysis of sheep's blood with the venom hæmolysin. No similar regular relationship of hæmolysis to cholesterol content is shown in the case of washed cells of different species and in many cases the quantity of lecithin present in the cells is much higher than in the whole blood. It is noted that not only is the cholesterol content of sheep's cells very high but their lecithin content is also lower than that of other species dealt with.

Where the cholesterol content is very high in relation to lecithin, cobra venom hæmolysis appears to be inhibited.

Further work which is in progress on the same subject shows that the proportionate hæmolysis caused by the cobra venom fraction varies in the same animal with the cholesterol content of the blood and in the case of monkeys infected with malaria it has been found that a gradual rise in percentage hæmolysis takes place during the progress of the infection with a subsequent return to normal, the cholesterol content showing an inverse relationship.

#### SUMMARY.

The hæmolytic action of cobra venom is associated with a fraction composed of globulin and primary proteose. It has been demonstrated that lecithinase accompanies this fraction.

Quantitative studies on cobra venom hæmolysis with this fraction on the blood of different animals have shown that the percentage hæmolysis of whole blood is roughly in inverse proportion to cholesterol content. No exact relationship has been shown between hæmolysis and relative content of cholesterol and lecithin within certain natural ranges, but where cholesterol is in large excess of lecithin, venom hæmolysis as a rule is slight in case of whole blood. This relationship is not shown in the case of washed cells where other factors may be concerned in the hæmolysis.

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## THE MIGRATION OF THE TOXIC CONSTITUENTS OF COBRA (*NAJA NAJA*) VENOM AT VARIOUS pH IN AN ELECTRIC FIELD.

BY

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[Received for publication, December 31, 1936.]

THE venom extracted from the poison glands of snakes contains a number of substances of more or less uncertain chemical composition. They can, however, be broadly classified into two groups: the proteins and the non-proteins. The former group constitutes the largest fraction of the dried venom (Ganguly and Malkana, 1936) and the toxins and the enzymes are always found associated with it. All attempts hitherto made to separate the toxins from the proteins have failed and this has led to the supposition that these substances are identical with proteins in their chemical nature. The possibility, however, that the toxins are held firmly adsorbed on the surface of protein particles should not be lost sight of. Since one of the important properties of proteins is that they possess characteristic iso-electric points, investigations were undertaken to ascertain whether the toxic constituents of snake venom also possess such iso-electric points. The results so far obtained with the neurotoxin and hæmolysin of cobra (*Naja Naja*) venom between pH 2 and pH 10 will be recorded in this paper.

### EXPERIMENT.

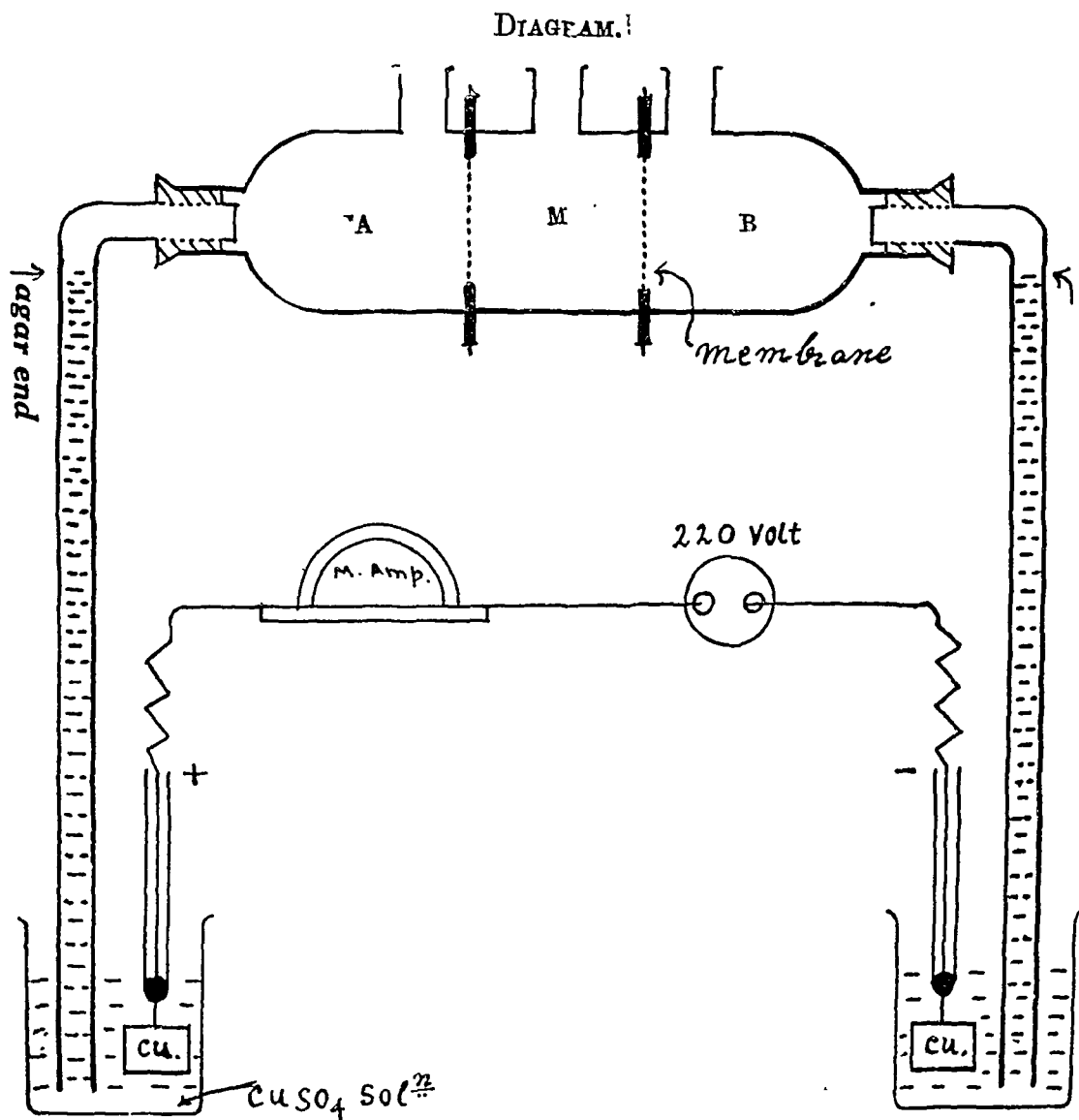
*The cataphoretic movement of neurotoxin and hæmolysin at different pH.*

One per cent solution of cobra (*Naja Naja*) venom was used in all the experiments recorded in this paper. A known weight of venom was dissolved in a suitable quantity of water and its pH adjusted to the desired value by adding drops of hydrochloric acid or caustic soda solution as the case may be. To this solution water and requisite buffer were added until the venom content of the solution was

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1 per cent. The amount of buffer added was such that every 10 c.c. of solution contained 9 c.c. of water and 1 c.c. of buffer solution. The buffer solutions were those prescribed by Sørensen. Phthalate, phosphate, and borate buffers were used between pH 2.2 and pH 5.0, pH 6.0 and pH 7.0, and pH 8.0 and pH 10.0, respectively. The toxicity of the venom was tested by intramuscular injection into pigeons. It was found that 0.4 mg. of venom (dry-weight) killed a pigeon weighing 300 g. to 310 g.

The apparatus used consists of a three-chambered glass vessel (see Diagram). The middle chamber is separated from the two side chambers by membranes of



suitable material. The solution of venom was placed in the middle chamber M and 10 c.c. of buffer solution were placed in each of the two side chambers A and B

which were put into electrical connection, by means of two agar bridges, with copper sulphate solution contained in two beakers. Two copper electrodes were immersed in the copper sulphate solution. One of the electrodes was connected directly to one pole of the 220 volt lighting circuit and the other electrode connected to the other pole through a milliammeter. The whole arrangement was made in such a way that experiments could be conducted inside a refrigerator maintained at 4°C. This precaution was taken to prevent the destruction of the toxins by heat developed during the passage of electric current. The membranes used in these experiments were those of parchment, cellophane, and the finest quality of the ultra-fine filter. The parchment and cellophane membranes usually obtained in the market contain pores of varying sizes. The pieces of membranes used by us were such that they did not allow water to ooze out through them in course of three to four hours when subjected to a pressure equivalent to that of a column of water 30 cm. high. After the passage of 216 coulombs of electricity through the cell extending over a period of five hours the contents of the cathodic and anodic chambers were tested for hæmolysin and neurotoxin. The presence of the latter was detected by ingestion into pigeons and that of the former by the hæmolysis of a suspension of red blood corpuscles of guinea-pigs containing a very small quantity of lecithin. The presence or absence of these toxins is indicated by plus and minus signs in the tables. It will be noticed from the data recorded in Tables I, II, and III that, while parchment membrane and finest quality of ultra-fine filter allow cobra-neurotoxin and hæmolysin to pass, the cellophane membrane completely stops their diffusion through it. The permeability of the membranes to these toxins is therefore in the order :—

Parchment > finest quality ultra-fine filter > cellophane.

It may also be noted that within the range pH 2.2 to pH 10.0 the neurotoxin as well as the hæmolysin migrate towards the cathode. Evidently therefore they do not possess any iso-electric point within the range of pH investigated. They are thus more strongly basic than some of the proteins such as hæmoglobin the iso-electric point of which is at pH 6.8.

TABLE I.

*Membrane used—parchment.*

pH.	NEUROTOXIN.		HÆMOLYSIN.	
	In cathode.	In anode.	In cathode.	In anode.
2.8 ..	+	—	+	—
4.2 ..	+	—	+	—
5.0 ..	+	—	++	—
6.0 ..	+	—	++	—
7.0 ..	+	—	++	—
8.0 ..	+	—	+	—
9.0 ..	+	—	+	—
10.0 ..	+	—	+	—

TABLE II.

*Membrane used—cellophane.*

pH.	NEUROTOXIN.		HÆMOLYSIN.	
	In cathode.	In anode.	In cathode.	In anode.
2·2 ..	—	—	—	—
4·2 ..	—	—	—	—
6·0 ..	—	—	—	—
8·0 ..	—	—	—	—
10·0 ..	—	—	—	—

TABLE III.

*Membrane used—finest quality of ultra-fine filter.*

pH.	NEUROTOXIN.		HÆMOLYSIN.	
	In cathode.	In anode.	In cathode.	In anode.
2·2 ..	+	—	+	—
4·2 ..	+	—	+	—
6·0 ..	+	—	+	—
8·0 ..	+	—	+	—
10·0 ..	—	—	+	—

*Purification of neurotoxin and hæmolysin by electrolysis.*—It has been shown in the previous section that the neurotoxin and the hæmolysin in cobra venom migrate towards the cathode between pH 2·2 and pH 10·0. Since different proteins have different iso-electric points it is probable that at a certain narrow range of pH

the majority of the proteins present in cobra venom will migrate towards the anode, while the neurotoxin and the hæmolysin will move towards the cathode, on application of an electric field. This suggests a possible method of partially separating the two toxins from the other proteins with which they are associated. Experiments were carried out to test how far such separation can be effected in actual practice. The experimental arrangement was the same as that described in the previous section, except that the solution of venom was kept in the anodic chamber, while the middle and the cathodic chambers were filled with buffer solution. Parchment membranes were used to separate the middle from the side chambers. In some experiments a combination of two membranes, that of parchment and the finest quality of ultra-fine filter, was used on the side of the middle chamber farthest from the anode. After the passage of 216 coulombs of electricity through the cells, the nitrogen, the neurotoxin, and the hæmolysin content of the middle chamber were estimated. The nitrogen was determined by the micro-Kjeldahl method. The neurotoxin was assayed by estimating the M. L. D. of pigeons weighing 300 g. to 310 g. by intramuscular injection. The hæmolysin content of the middle chamber was expressed in an arbitrary unit. It was assumed that one g. of dry cobra venom contains 2,500 units of hæmolysin. The quantity of solution of the middle chamber required to produce complete hæmolysis of a given suspension of guinea-pig's red blood corpuscles containing a fixed quantity of lecithin, in a given time, say 15 minutes, was determined. Then the amount of cobra venom which will be required to produce complete hæmolysis of an identical suspension of guinea-pig's red blood corpuscles in the same time, i.e., 15 minutes, was determined by a series of trial experiments. From these data we can calculate the number of units of hæmolysin contained in the total volume of the solution of the middle chamber in terms of the arbitrarily chosen value of the hæmolysin content of one gramme of dried cobra venom. The results obtained are recorded in Tables IV and V. Since the M. L. D. of a pigeon is 0.4 mg. of dry cobra venom and since this amount of venom contains 0.06 mg. of nitrogen, therefore the number of M. L. D. per mg. of nitrogen of the dry venom is 16.6. The figures in the fifth column are obtained by dividing the corresponding figures in the fourth column by 16.6. It may be noted from the data recorded in Table IV that as the pH increases the hæmolysin content of the middle chamber increases and reaches a maximum at pH 6.0. After this point it decreases again as the pH increases still further. The maximum purification of hæmolysin is, however, effected at pH 7.0 where the proportion of protein is reduced to less than one-third of the amount present in the original (dried) venom. Similarly from Table V it appears that the neurotoxin content of the middle chamber increases at first, reaches a maximum at pH 4.2 and then diminishes again as the pH increases to a still higher value. The maximum purification of neurotoxin occurs at pH 4.2. The extent of purification is almost the same as that of hæmolysin at pH 7.0. As some of the hæmolysin and neurotoxin pass from the middle to the cathodic chamber when the separating membrane between the two is only parchment, some experiments were carried out using a combination of parchment and the finest quality of the ultra-fine filter separating the middle from the cathodic chamber. These results are recorded in Table VI. Although by this arrangement the concentration of neurotoxin in the middle chamber can be considerably increased, yet the purification effected is not greater than the maximum value 3.4 times, recorded in Table V, at pH 4.2.

TABLE IV.

*Membrane used—parchment.*Number of units of hæmolysin per mg. of N<sub>2</sub> of dry cobra venom = 16.6.

pH.	TOTAL CONTENT OF THE MIDDLE CHAMBER.		Number of units of hæmolysin per mg. of N <sub>2</sub> in the middle chamber.	Purification effected (times).
	N <sub>2</sub> in mg.	Number of units of hæmolysin.		
2.8 ..	0.78	18	23.07	1.4
3.4 ..	0.56	20	35.71	2.1
4.2 ..	0.528	26	49.24	3.0
5.0 ..	0.58	27	46.54	2.8
6.0 ..	0.664	30	45.18	2.7
7.0 ..	0.36	20	55.55	3.3

TABLE V.

*Membrane used—parchment.*Number of M. L. D's of neurotoxin per mg. of N<sub>2</sub> of dry cobra venom = 16.6.

pH.	TOTAL CONTENT OF THE MIDDLE CHAMBER.		Number of M. L. D's of neurotoxin per mg. of N <sub>2</sub> in the middle chamber.	Purification effected (times).
	N <sub>2</sub> in mg.	Number of units of neurotoxin.		
2.8 ..	0.78	20	25.64	1.5
3.4 ..	0.56	20	35.71	2.2
4.2 ..	0.528	30	56.81	3.4
5.0 ..	0.58	20	34.48	2.1
6.0 ..	0.664	22	33.13	2.0
7.0 ..	0.36	17	47.2	2.8

TABLE VI.

*Membrane used—parchment and ultra-fine (finest) filter at the cathode side, parchment only at the anode side.*

Number of M. L. D's of neurotoxin per mg. of N<sub>2</sub> of dry cobra venom = 16.6.

pH.	TOTAL CONTENT OF THE MIDDLE CHAMBER.		Number of M. L. D's of neurotoxin per mg. of N <sub>2</sub> in the middle chamber.	Purification effected (times).
	N <sub>2</sub> in mg.	Number of M. L. D's of neurotoxin.		
6.0 ..	0.784	40	51.02	3.1
7.0 ..	0.622	33	53.05	3.2
8.0 ..	0.556	14	25.18	1.5
9.8 ..	0.560	14	25.0	1.5

*Separation of neurotoxin from hæmolysin.*—From an examination of the data recorded in Tables IV, V, and VI it appears that maximum purification of neurotoxin and hæmolysin occurs at different pH. This suggests that their cataphoric velocities do not change with pH in the same way. It should, therefore, be possible to effect a partial separation of the two substances by electrolysis at different pH. To test this point experiments were carried out with the apparatus described in the previous section. The experimental procedure was the same as before. The results are recorded in Table VII. Since the ratio of neurotoxin to hæmolysin in the original dry cobra venom is *one* according to the arbitrarily chosen unit of hæmolysin, it follows that rise in the value of this ratio above *one* would indicate a relative enrichment of the neurotoxin fraction. Correspondingly, a fall in the ratio below *one* would mean an enrichment of the hæmolysin fraction. It will be noticed from the data in Table VII that there occurs a partial separation of the two by this method. The maximum concentration of hæmolysin relative to neurotoxin occurs between pH 5.0 and pH 6.0 and that of neurotoxin relative to hæmolysin at pH 4.2.

TABLE VII.

pH.	TOTAL CONTENT OF THE MIDDLE CHAMBER.		Ratio.
	Number of M. L. D's of neurotoxin.	Number of units of hæmolysin.	
2.8 ..	20	18	1.11
3.4 ..	20	20	1.0
4.2 ..	30	26	1.15
5.0 ..	20	27	0.75
6.0 ..	22	30	0.73
7.0 ..	17	20	0.85

# SUMMARY.

(1) It has been observed that while parchment membrane and the finest quality ultra-fine filter are permeable to cobra-neurotoxin and hæmolysin, the cellophane membrane is impermeable to them. The permeability of the membranes are therefore in the order : parchment > finest ultra-fine filter > cellophane.

(2) The hæmolysin and neurotoxin have no iso-electric point between pH 2.2 and pH 10.0. It appears, therefore, that they are fairly strongly basic in their chemical character.

(3) By cataphoretic experiments at various pH, using intercepting membranes, it has been possible to reduce the quantity of proteins associated with the hæmolysin and neurotoxin to less than one-third of their original value.

(4) It has been shown that partial separation of the hæmolysin from the neurotoxin can be effected by cataphoretic experiments at different pH, in which intercepting diaphragms were used.

# ACKNOWLEDGMENTS.

In conclusion we thank very warmly the authorities of the Bengal Immunity, Calcutta, for facilities offered for further research on this problem. We also thank the Director of the Haffkine Institute, Bombay, for kindly supplying the venom at a reduced price.

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## A FORECAST OF POPULATION IN INDIA AT THE CENSUS OF 1941\*.

BY

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FORECASTS of population in India are difficult for several reasons. In his recent book Carr-Saunders (1936) points out that the application of the usual methods of forecast may not be justified when dealing with India. He states, 'It is not possible to forecast the future population of India on lines similar to those employed when dealing with other countries; for there is no question of projecting into the future present birth and death rate trends with appropriate modifications'. He points out that the position in India is in marked contrast with that of the Western countries and Japan. In the latter 'the population has been raised above the immediate impact of natural forces, and the vital statistics, even in those cases where there is evidence of congestion of population, are not affected by the vagaries of climate and the yield of harvests'. India, on the other hand, continues to be so in spite of an expansion of irrigation and cultivated land because the growth of population has kept pace with the increase of food supply. Though famines on a large scale have been less frequent than formerly, a failure of the monsoons is followed by widespread and acute distress and he points out that 'the high mortality during the bad seasons has always been due mostly to epidemics which are enabled to spread on account of the enfeebled condition of the population consequent on lack of sufficient food'. The bad periods have also been marked, he notes, by a fall in the birth rate below the average. 'The future population of India will, therefore, be governed by such expansion of opportunities for subsistence as may take place.'

The picture that Carr-Saunders has presented represents the true position. But what he has in mind is a long-term prediction of India's population. I propose to confine myself to the present decade, 1931-1941, in the hope that the margin of error may be reduced to some extent. Further, in spite of its imperfections, I am

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\* First read at the Indian Population Conference, Lucknow, February 1936, and slightly modified afterwards.

compelled to make use of the statistical method in order to avail myself of objective standards for the measurement of growth. In doing so, however, I shall take note of the nature and quality of the material that Indian vital statistics present.

The number of censuses in India has been only seven, so that the projection of past experience into the future becomes unsatisfactory because of the narrow range of such experience. A second reason is that the six intercensal periods, for which population growth is known, have been characterized by extremely variable rates of growth.

TABLE I.

*Actual rates of growth in India during intercensal periods.*

Period.	Rate per cent* of real increase.
1872-1881 .. ..	1.5
1881-1891 .. ..	9.6
1891-1901 .. ..	1.4
1901-1911 .. ..	6.4
1911-1921 .. ..	1.2
1921-1931 .. ..	10.6

\* Quoted from the *Actuarial Report, Census of India, 1931, 1, Part I* (Vaidyanathan, 1931).

Every alternate intercensal period has shown a very low rate of increase. The causes for these low rates were extensive famines during the periods 1872-1881 and 1891-1901 and, during 1911-1921, the pandemic of influenza, which swept the country in 1918-19 and carried away about 14 millions, accounted for the smallness of the increase. The alternation of low with relatively high rates of increase cannot, as the above table may suggest, be accepted as a feature of population growth in India. However, in attempting to predict the probable population, any method of extrapolation employed becomes unsatisfactory because of these widely differing rates from census to census.

For forecasting population various methods have been used. When growth is not characterized by violent fluctuations, short-term predictions of sufficient accuracy can be obtained by the use of equations which have no justification except that they describe past events satisfactorily. Secondly, if the vital statistical records are fairly accurate, mortality rates for specific ages and the two sexes form sufficiently stable material for building up the structure of population in the immediate future. Again, Raymond Pearl and his co-workers have elaborated certain formulæ which, they consider, represent the biological law of growth.

In regard to India, the history of population growth has, as already stated, been characterized by violent changes. Again Indian vital statistics are so unsatisfactory that specific mortality rates can hardly be used with confidence for

making a prediction. Under the circumstances, what has been attempted in this short paper is to make an assessment of the trend of those events which influence population growth and thus arrive at a forecast of the probable population in 1941. Incidentally, Pearl's symmetrical logistic curve was also fitted to the populations of India at the respective censuses and the expected population of 1941 by this method was also calculated. But, in view of what has been stated later, I am not inclined to attach much value to this estimate.

In a paper 'Probable Trend of Population Growth in India' (Raja, 1935), I have shown that the age composition of the married female population of India at the reproductive ages, 15 to 50 years, as enumerated at the 1931 census, was more favourable to growth of population than that at each of the three previous censuses of the present century. I have also shown that the expectation of life for girls born in 1931 during their reproductive period was higher than for girls born at the previous censuses to the extent of about one and a half years. While the latter may not be expected to influence population growth till after 1946, the former is likely to enhance the rate of growth during 1931-1941.

The growth of population in a country is determined by four factors, the birth and death rates and the figures for immigration and emigration. As regards migration, it has ceased to be of any great importance in view of increasing restrictions that are being placed on the admission of Indians in other countries. During the decade 1921-1931 the outward balance of migration was only about a million and three quarters or only about 5 per cent of the total increase of nearly 34 millions during these ten years. The birth rate in British India, for which alone I have figures, has been more or less steady from the beginning of the century, while the death rate has shown a tendency to decline during the decade 1921-1931. The consequence is that the natural increase of population, which is the balance of births over deaths, has, on the whole, been on the increase within the last ten or twelve years.

TABLE II\*.

Period.			Birth rate per mille.	Death rate per mille.	Annual excess of births over deaths.
1901-1911	..	..	38	34	933,623
1911-1921	..	..	37	34	667,654
1921-1931	..	..	35	26	1,995,301
1926	..	..	35	27	1,935,069
1927	..	..	35	25	2,506,977
1928	..	..	37	26	2,702,459
1929	..	..	36	26	2,297,950
1930	..	..	36	27	2,207,265
1931	..	..	35	25	2,520,791
1932	..	..	34	22	3,248,840
1933	..	..	34	22	3,582,089

\* The figures have been quoted from the *Annual Report of the Public Health Commissioner with the Government of India* (1933).

The tendency for a fall in the death rate is clear. In the accompanying graphs (Plates XXXIX and XL) are presented the specific mortality rates at ages for males and females in British India for the period 1921-1933 smoothed by fitting straight lines. The rates are not those given in the Public Health Commissioner's reports because the latter, except for 1932 and 1933, were always calculated on the previous census populations howsoever remote the census year was. Hence, in the rates presented in these graphs, I have corrected them for growth of population during the intercensal period. The specific death rates are bound to be incorrect in view of mis-statements of age but the trend of mortality is downwards at all age groups except the last one, 60 years and upwards, in the case of both sexes. Therefore the suggestion is that the fall in mortality shown in recent years is a real one and that the death rate is not likely to show an upward trend at least in the near future. This assumption is strengthened by the fact that, in British India, to which these figures relate, the population aged 60 and upwards forms only a very small proportion of the total, while about 50 per cent of both sexes are between the ages 15 to 50 years, during which the rates of mortality are lower than at either end of life.

TABLE III.

*Population for registration area in British India (1931 census).*

Ages.	Males (percentage of total).	Females (percentage of total).
0-5 .. ..	39.45	39.25
15-50 .. ..	50.70	50.73
50-60 .. ..	5.88	5.80
60 and upwards ..	3.97	4.22
TOTALS ..	100.00	100.00

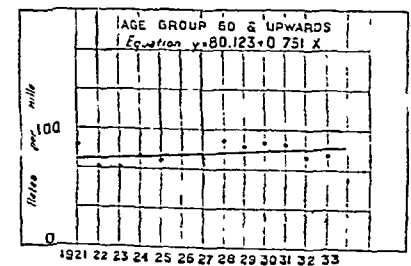
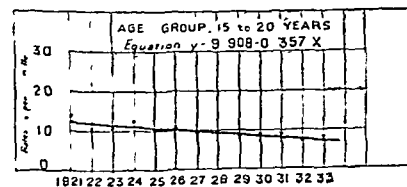
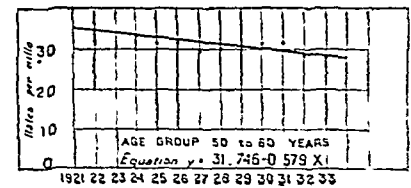
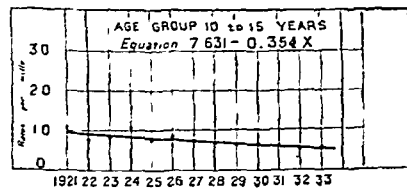
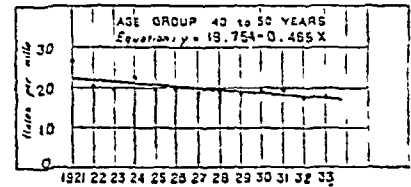
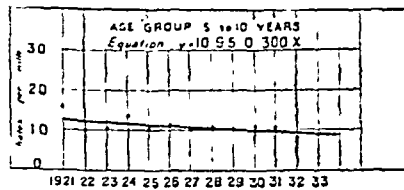
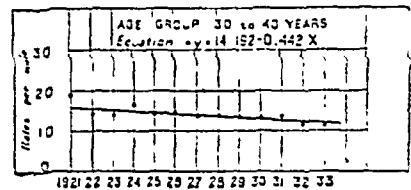
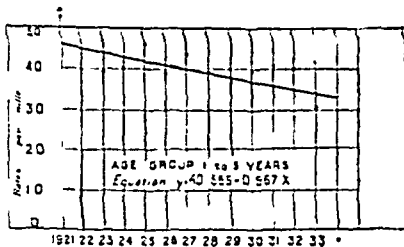
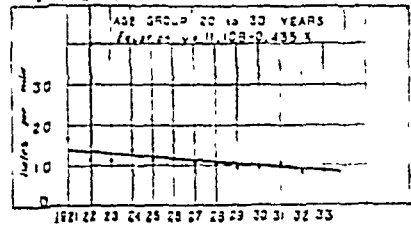
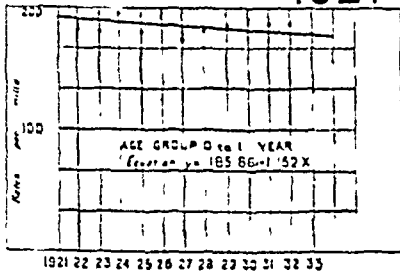
For attempting a prediction of the probable population of India in 1941 the figures for natural increase or balance of births over deaths are available for British India for the period 1921-1933. To these figures curves of increasingly higher order can be fitted by a method given by R. A. Fisher, in which at each stage the gain in closeness of fit to the given data can be evaluated. This was carried out up to the fourth degree regression equation and it was found that a straight line and second order parabola gave better fits than the higher ones, the second order parabola being better than the straight line. The straight line trend gave 36,881,353 as the total expected increase in population between the census of 1931 and that of 1941. The censuses have been taken to be at the end of February in both cases. That of 1931 was on 26th February. The second order parabola gave 35,438,776 as the natural increase. The difference between the two estimates is only about a million and a half. Now these figures for increase relate to a population of 265,688,864

# SPECIFIC MORTALITY RATES BY AGES

for MALES in

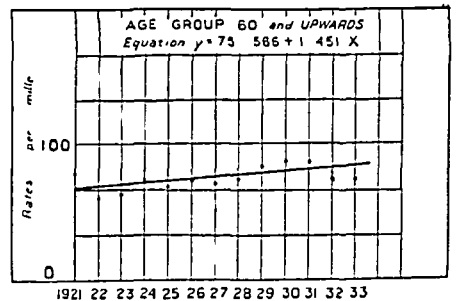
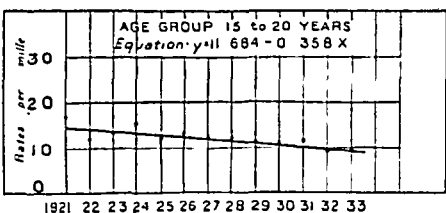
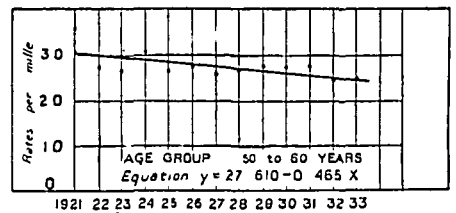
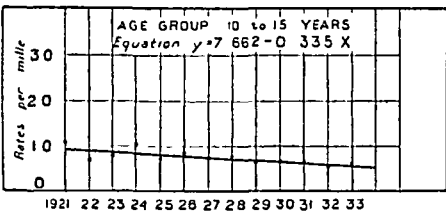
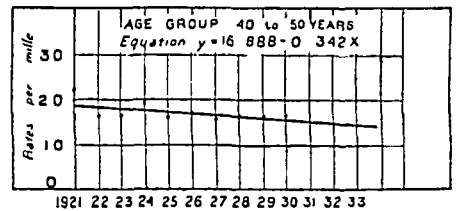
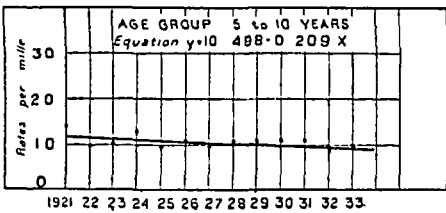
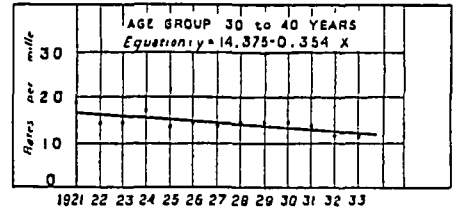
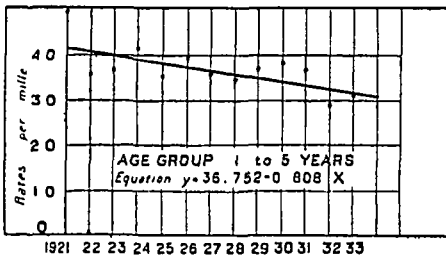
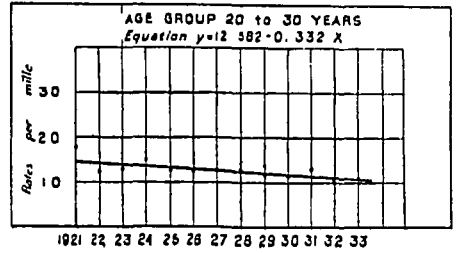
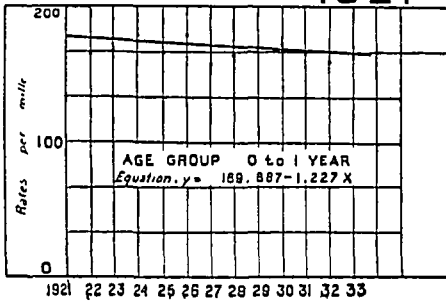
## BRITISH INDIA FOR THE PERIOD

### 1921 to 1933



# SPECIFIC MORTALITY RATES BY AGES

for FEMALEs in  
BRITISH INDIA FOR THE PERIOD  
1921 TO 1933



which represents, according to the Public Health Commissioner, the revised estimate for the population of the registration area of British India at the census of 1931. If we make the assumption that the rate of growth in British India is applicable to the whole of India, then the population of India in 1941 is likely to be 401,816,628 and 399,900,869 by the straight line and second order parabola respectively. The inference is that the population of India is likely to be approximately 400 millions at the 1941 census. This represents an increase of about 13.4 per cent during the present intercensal period.

It is recognized that the methods used for forecast are lacking in fineness. But the nature of the figures we are dealing with is such that it is doubtful whether they admit of finer forms of treatment.

One defect of this method of estimation is that registration of births and deaths are both defective in India. There is reason to believe that it has improved within the period to which the figures of this estimate relate, mainly owing to the activities of the Public Health Departments which came into being in most of the Provinces after the Reforms of 1919. It may be expected that failure of registration in the case of both births and deaths will, to some extent, counterbalance each other, though it is impossible to form an idea of the extent to which such counterbalancing takes place. Further, the period 1921-1933 for which we have taken the figures for natural increase in predicting what is likely to happen in the succeeding years of the present intercensal period, has been characterized by no very violent fluctuations and hence the extension of this experience is perhaps justified.

Pearl's symmetrical logistic curve was fitted to the enumerated populations of India. Pearl claims that the logistic curve represents the biological law of population growth. Others have contested this claim. As pointed out by Hogben (1931) the logistic curve 'is characterized by an initial phase of slow growth, a succeeding stage of rapid increase, and a final phase of declining increase towards a stable limit. It is, therefore, suited to describe populations displaying slow, rapid, increasing or diminishing growth rates; and it is not surprising that census statistics can generally be fitted on to some portion of a curve which possesses such catholic characteristics'. Pearl has, in fact, shown that the populations of many countries are given a good fit by his logistic curve. This is to be anticipated in view of the nature of the curve. But it does not show that the curve expresses a biological law of growth for human populations. Hogben reproduces the figures for the population of France calculated by Bowley by fitting a second order parabola and, side by side, the figures given by Pearl's logistic curve. Bowley's figures give a better fit to the actuals than Pearl's, a fact which shows that the logistic can claim no unique fit for representing the growth of human populations.

Pearl's critics also point out that his finding about density being negatively correlated with fertility, mainly deduced from experiments on *Drosophila melanogaster*, cannot be applied to human populations, as low fertility in man, at least in Europe and America, seems to be associated with those social classes least subject to overcrowding and the adverse effects resulting from it. In human communities the causes of low fertility seem to be different. Stevenson (1920)

has shown, by his analysis of English fertility rates for social classes, that the causes that are operative are volitional acts of man interfering with the natural process of reproduction. Further, Carr-Saunders (1922) has produced considerable evidence to show that while, in animals, fertility is highly correlated with fecundity or the absolute power of reproduction, in man, from the earliest evidence available, the indications are that he has attempted to interfere with the full realization of his fecundity.

It is therefore doubtful whether the laws of population growth as deduced from experiments on drosophila and yeast cells are applicable to human communities. The logistic curve was, however, made use of in this place because it represents a relatively simple method of prediction for the immediate future and it was therefore desirable to see whether it would give a forecast similar to the one we have already arrived at on a different set of considerations.

In fitting the curve, we must correct the enumerated populations for additions of territory at the different censuses and for improvements in methods of census operations. These corrected figures can alone give us an estimate of the rate of growth during the period. The following are the corrected populations in millions which I have used for fitting the logistic curve:—

TABLE IV.

Census year.	Population in millions.
1872 .. ..	206·162
1881 .. ..	209·162
1891 .. ..	233·462
1901 .. ..	237·562
1911 .. ..	256·262
1921 .. ..	259·962
1931 .. ..	293·857

These populations have been calculated from the two tables given on page 143 of the *Census of India*, 1931, 1, Part I.

The lower limit was taken at 150 millions and the upper limit at 600 millions. Greenwood (1925) has shown that, when curves like Pearl's logistic are used, 'no great importance attaches to the precise figure for the ultimate population'. He showed that, for England and Wales, his own upper limits of infinity and 55·56



millions as well as Pearl's upper limit of 70·67 millions 'gave very fair reproductions of the facts'. The only relationship that need hold between the two limits is that the logarithms of the  $Z$ 's\* which are calculated should, for the symmetrical logistic, lie approximately on a straight line. The two limits of 150 and 600 millions gave values of  $Z$  whose logarithms lay approximately on a straight line as shown in Diagram 1:—

$$\text{Log } z = 0.608,879,9 - 0.008,521,7 t.$$

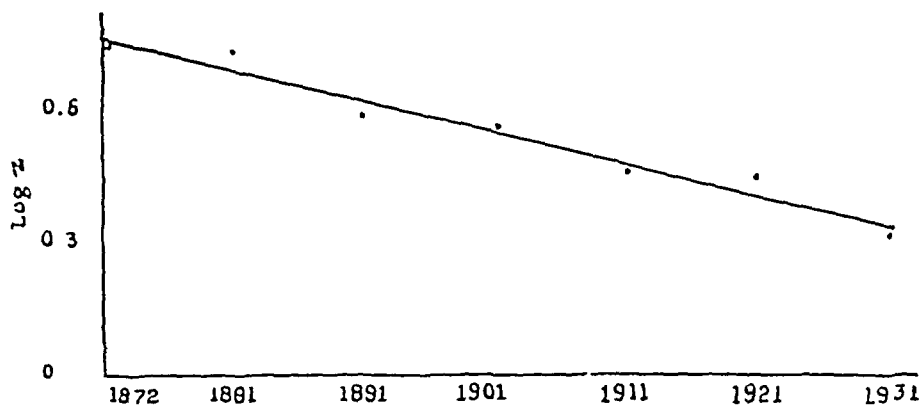


DIAGRAM 1.

We may test whether the regression straight line has given a good fit to the points.

*Analysis of variance.*

Source of variation.	Degrees of freedom.	Sum of squares.	Mean square.
(1) Regression straight line ..	1	0.198	0.198 ( $v_1$ )
(2) Residual .. ..	$\frac{5}{6}$	$\frac{0.007}{0.205}$	0.001 ( $v_2$ )

$$\frac{v_1}{v_2} = 198; n_1 = 1; n_2 = 5; P \text{ is less than 1 per cent.}$$

The regression straight line has given a better fit than the horizontal straight line through the mean. The small value for the residual sum of squares also suggests that there has been a good fit.

\*  $Z = \frac{l-y}{y-d}$  where  $l$  and  $d$  are the upper and lower limits respectively and  $y$  the population at each census.

# 1190 *A Forecast of Population in India at the Census of 1941.*

The equation of the symmetrical logistic curve is:—

$$Y-150 = \frac{450}{1 + 4.063, 31e^{-0.019,622t}}$$

$t_0$  corresponds to 1901, time being measure in years, so that 1872 corresponds to  $t_{-29}$  and 1931 to  $t_{+30}$ .

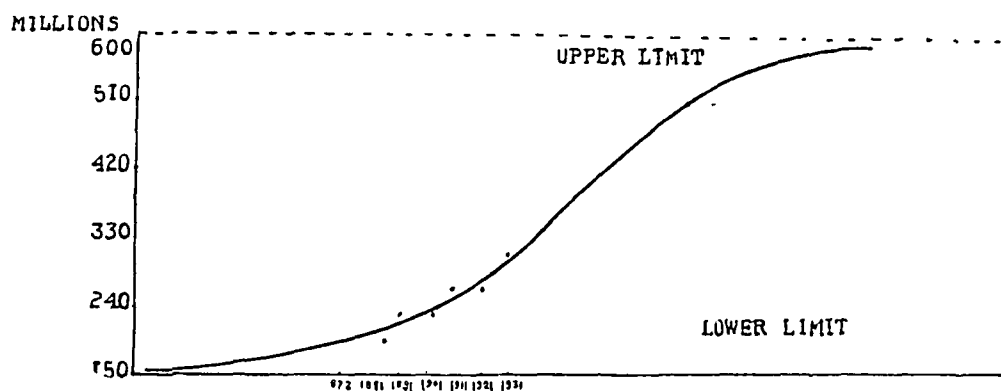


DIAGRAM 2.

The enumerated and calculated populations are shown below:—

(1)	(2)	(3)	(4)
Census year.	Enumerated population.	Calculated population (logistic curve).	Difference between (2) and (3) as percentage of (2).
1872 ..	206.162	205.025	—0.552
1881 ..	209.162	214.138	+2.379
1891 ..	233.462	225.872	—3.251
1901 ..	237.562	238.875	+0.553
1911 ..	256.262	253.262	—1.171
1921 ..	259.962	270.180	+3.931
1931 ..	293.857	288.231	—1.915

Column 4 gives the deviations of the calculated populations from the enumerated ones expressed as percentages of the latter. If these percentages are summed

irrespective of their signs, the mean deviation is seen to be 1·965 per cent. The logistic curve has given a fairly satisfactory fit. Using the same equation, the population of 1941 is 307·697. On this rate of increase the enumerated population of 1931 becomes 376,667,068 in 1941.

The logistic curve has given a lower rate of growth, namely 6·75 per cent, than that given by the previous method. It is likely to be an under-estimate. The Public Health Commissioner has shown in his *Annual Report* for 1934 that the estimated population for the middle of that year gave an increase of about 3·8 per cent for British India. This increase relates to a period of about three years and four months after the census on 26th February, 1931. If this rate holds good for India as a whole, the expected increase during 1931–1941 becomes approximately 11·4 per cent, which agrees more closely with the estimate given by the first method than that given by the logistic curve. When the logistic curve is fitted to our data we are taking into account the very low rates of increase in each of the alternate intercensal periods and this may perhaps explain the low rate of growth predicted for the present intercensal period. Under the circumstances I am not prepared to attach any great importance to the forecast given by the logistic curve. If the present decennium should be free from abnormal visitations of epidemics or famines I think that the enumerated population of 1941 is likely to approximate to 400 millions.

I have to acknowledge my thanks to Mr. K. C. Basak, Research Assistant in this Department, for having kindly checked the calculations in this paper.

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